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Influence of Asphyxia on Membrane Potential Level and Action Potentials of Spinal Moto- and Interneurons.

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Abstract.

KOLMODIN, G. M. and C. R. SKOGLUND. Influence of asphyxia on membrane potential level and action potentials of spinal moto- and interneurons. *Acta physiol. scand.* 1959. *45.* 1—18. — Asphyxial changes in spinal moto- and interneurons of decapitate cats were studied by means of intracellular microelectrodes. In both types of neurons the first change in membrane potential was usually observed after about 30 sec in the form of a depolarization of 5—10 mV which after some 30 sec was followed by a phase of more rapidly developing depolarization of larger amplitude; during recovery a phase of increased polarization may be observed. Interneurons showed signs of being more resistant to asphyxia than motoneurons. The spike potential underwent a successive diminution in amplitude, at first owing to the decrease in membrane potential value, later also due to a change in spike peak level. During the later stage of asphyxia a decrease of both the rate of rise of the ascending and the rate of decay of the descending spike phases could be

demonstrated. Rhythmical interneuron discharges showed periods of increased and decreased frequency before final blocking.

The effects of asphyxia and anoxia on synaptic transmission in the spinal cord have been extensively studied by various methods, and different problems, from the site of asphyxial block in a given reflex pathway (BROOKS and ECCLES 1947) to the differential vulnerability of various reflex systems (*e. g.* KIRSTEIN 1951, GELFAN and TARLOV 1955) have been taken up for study. A review of earlier investigations has been givenby KIRSTEIN.

The basic mechanism of the asphyxial cell action in terms of membrane potential changes has been approached by VAN HARREVELD (1946) by studying the slow depolarization processes in the grey matter and by LLOYD (1953) in an ingenious analysis of antidromic ventral root electrotonus. The recent development of the microelectrode technique evidently offers opportunities for a direct recording of the membrane potential changes in various types of cells in the spinal cord.

The present paper is limited to a description of some typical effects of brief periods of asphyxia on the membrane potential level and the action potential of motoneurons and — on a smaller scale — of interneurons in the cat's spinal cord.

Methods.

Spinal, curarized cats kept on artificial respiration were used for the experiments, and asphyxia was achieved by disconnecting the pump from the tracheal cannula. Laminectomy was performed in the lumbosacral region. In all experiments the ipsilateral ventral roots from L_4 to S_1 were arranged for antidromic stimulation. In some experiments dorsal roots were used for afferent stimulation, in others peripheral nerves.

As a detailed description of the recording and stimulation technique used has been given by HAAPANEN, KOLMODIN and SKOGLUND (1958), only a few principal data will be summed up here. The recording was made via capillary electrodes filled with 2.7—3.0 M KCl solution which were connected to an input stage of cathode follower type (HAAPANEN and OTTOSON 1954). A direct coupled amplifier deflected one beam of a double-beam oscilloscope, the other beam of which was used for indication of zero level or time. An inkwriter (Siemens, type SD 12 K, response time 1/sec) for continuous recording of the membrane potential was also connected to the amplifier.

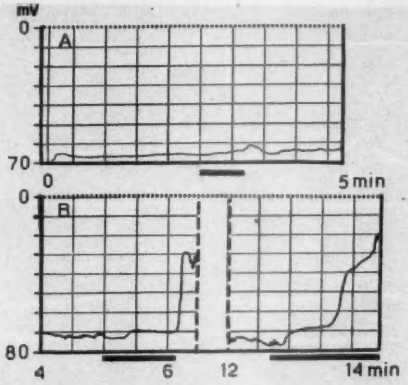

Fig. 1. Changes in membrane potential levels of motoneurons due to asphyxia of varying duration (marked by *thick lines*). *A*, impalement of neuron at beginning of record followed by typical initial decay of membrane potential before steady level is reached; asphyxial period 40 sec. *B*, another motoneuron, repeated asphyxiation during 70 and 110 sec. Between *dotted vertical lines* part of record cut out; course of recovery after first depolarization therefore not shown. Time in minutes represents total period of continuous recording. Time between vertical lines 30 sec.

Results.

I. Asphyxial changes in motoneurons.

The first observed effect of asphyxia on motoneurons is a decrease of the membrane potential level. It is difficult to determine the exact onset of this slowly developing change — especially since normally slight fluctuations in the potential level often occur — but usually the depolarization becomes quite evident 30—40 sec, occasionally as early as 20 sec, after cessation of respiration (Fig. 1 *A*). Obviously great latency variations are to be expected, considering the varying state of different preparations with respect to circulation and respiration and other factors influencing the condition of the nerve cells.

The increase of the asphyxial depolarization with time usually occurs in a characteristic stepwise way. An initial plateau of rather moderate depolarization, of 5—10 mV, is often maintained for about 30 sec, after which a second phase of a more rapidly developing depolarization sets in (Fig. 1 *B*; Fig. 3).

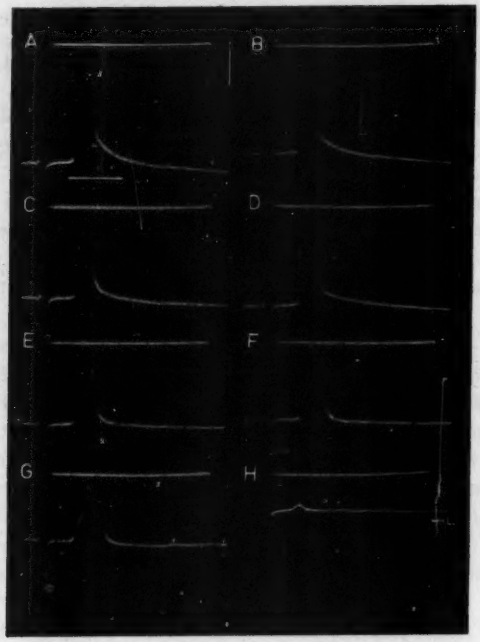

Fig. 2. Influence of asphyxiation on action potentials of antidromically activated motoneuron. *A* before asphyxia, *B* 30 sec, *C* 75 sec, *D* 120 sec, *E* 135 sec, *F* 138 sec, *G* 141 sec and *H* 144 sec after cessation of ventilation. *Upper beam* at zero level. Vertical bar 20 mV. Time bar 5 msec.

If ventilation is restored during the first phase the motoneuron may quickly repolarize again, as shown in Fig. 1 *A*, but after longer periods of asphyxia, as in Fig. 1 *B*, the second phase develops in spite of readmission of oxygen. In the first test the final depolarization plateau represented a reduction of more than 50 per cent of the initial membrane potential value. The cell did not attain its full membrane potential value until after 5 minutes of reventilation. There seem to be great variations in the course of the postasphyxial repolarization; this phase has, however, not been systematically analysed in this work.

When the motoneuron of Fig. 1 *B* was again exposed to asphyxia it reacted after a somewhat shorter latency but in fundamentally the same way. The asphyxial period was longer this time, resulting

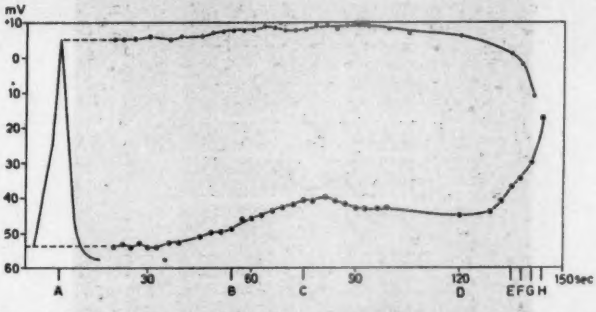

Fig. 3. Graphical representation of successive changes in membrane potential level (*large filled circles*) and spike peak level (*small filled circles*) of antidromically activated motoneuron during a 150-sec period of asphyxiation. Same experiment as Fig. 2 whose records were taken at intervals marked by corresponding letters on the time scale. Time in sec from cessation of ventilation.

in a depolarization to a final level of about 20 mV. In this case no recovery was observed during the first 5 minutes of reventilation.

Another example of such a typical depolarization in a motoneuron, resulting from a 2.5-minute period of arrested respiration, is given in Figs. 2—3, selected to show also the concomitant changes in the antidromically evoked action potential. Record *A* in Fig. 2 is a control picture, illustrating an antidromic spike which takes off from a membrane potential level of 55 mV, shows a characteristic notch on the rising phase (cf. BROCK, COOMBS and ECCLES 1953) at about 25 mV and overshoots the zero line by about 5 mV. A schematic drawing of this potential is found to the left in Fig. 3 which shows the successive changes in membrane potential level and spike peak level during asphyxia. During the first plateau of depolarization, corresponding to a lowering of the membrane potential by about 10 mV, no significant variations occur in the spike peak level, whereas during the later phase of the more rapid drop of the membrane potential (from 120 sec) there is a pronounced change in the spike peak level.

Records *B—H* in Fig. 2 have been selected from various moments of the asphyxial period (cf. corresponding letters in the time scale in Fig. 3). It appears from these records that parallel with the lowering of the membrane potential there are significant changes in the slow repolarization ("afterpotentials") following the

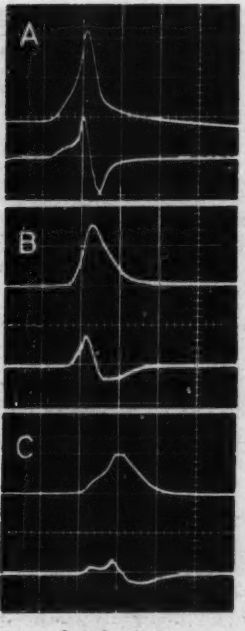

Fig. 4. Influence of asphyxiation on ascending and descending spike phases of antidromically activated motoneuron. *A* before asphyxia, *B* 135 sec, *C* 141 sec after stop of respiration. *Upper curves* in records obtained by photoelectric scanning of paper-masks of potentials obtained in experiment Fig. 2. *Lower curve* in each record represents the first derivative of the upper one. Time between vertical lines 1 msec. Maximum rate of rise in *A* 100 m/sec.

fast falling phase of the spike; thus the repolarization phase below the initial membrane potential level in *A* (record shows only first part) becomes successively smaller in *B*—*D* and is missing from *E* on.

The shape of the spike also undergoes characteristic changes. Thus for instance in record *E* the rising spike phase seems to be slowed down both below and above the notch; in *G* these changes are quite obvious and result in a more pronounced notch and a broadening of the spike potential. By means of a photoelectric function generator (SUNSTEIN 1949, cf. also HAAPANEN, KOLMODIN and SKOGLUND 1958) some potentials from this series have been

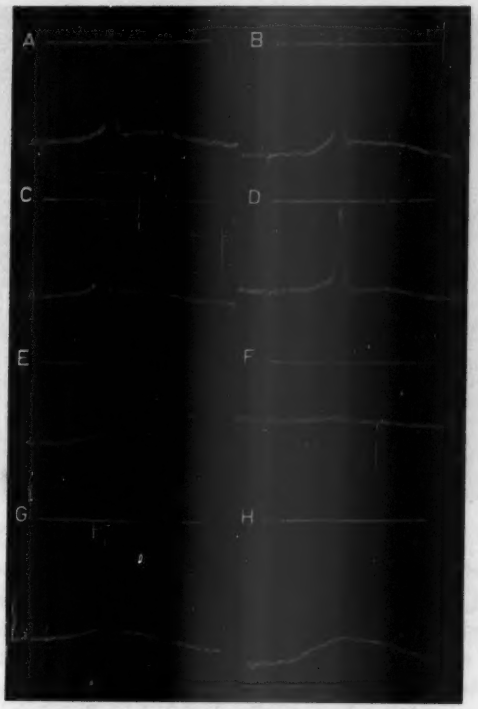

Fig. 5. Asphyxial and postasphyxial changes in orthodromically activated motoneuron. *A* before asphyxia; *B* 57 sec, *C* 72 sec, *D* 75 sec, *E* 78 sec and *F* 81 sec after cessation of ventilation; *G* 100 sec and *H* 112 sec after restoration of ventilation. *Upper beam* at zero level. Vertical bar 20 mV. Time bar 5 msec.

reproduced (upper curves in Fig. 4) and the first derivative has been obtained by passing the potentials through an amplifier of short time constant (lower curves). In Fig. 4, record *A* is a control picture before asphyxia, showing the different rate of rise of the two parts of the rising spike phase. In record *B*, after more than 2 minutes' asphyxia, the maximum rate of rise can be seen to be lowered by about 30 per cent. The change in the falling phase is also obvious. In the last record, *C*, at a somewhat later stage of asphyxia, the rate of change of both the rising and falling phases has decreased to about 30 per cent of the initial values.

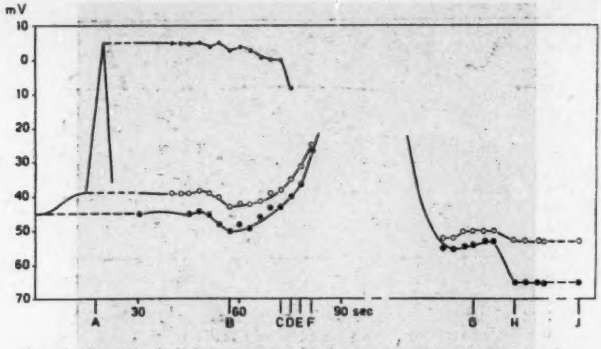

Fig. 6. Graphical representation of successive changes in membrane potential level (*large filled circles*), firing level or prepotential maximum (*open circles*) and spike peak level (*small filled circles*) of orthodromically activated motoneuron during and after an 85-sec period of asphyxiation. Same experiment as Fig. 5 (cf. letters on time scale). Time in sec from arrest of respiration; *G* 100 sec, *H* 112 sec and *J* 140 sec from restoration of ventilation.

A corresponding series of records from an orthodromically activated motoneuron is illustrated in Fig. 5. Record *A* is a control picture of the multisynaptically activated motoneuron with its characteristic slowly rising prepotential from which a spike potential takes off at a critical value (of about 40 mV). The spike in *A* is schematically depicted in Fig. 6, in which the changes in membrane potential, firing level and spike peak level during the period of asphyxia have been plotted against time. After a fairly short period of increased polarization which definitely supersedes the normal variations, the final drop of the membrane potential occurs after about 75 sec of asphyxia. Until then the spike remains fairly constant but after a brief phase of successive decline it suddenly drops out. As seen from the oscillographic pictures in Fig. 5 the spike form undergoes the same changes as the antidromic spike, *i. e.* both rising and falling phases become successively slower, resulting in a broadening of the spike, although not so pronounced as in Fig. 2. The absolute value of the firing level is also successively lowered parallel with the decrease of the membrane potential, but also in record *E* there is still a small prepotential visible although no spike is fired at this membrane potential level.

Records *G* and *H* in Fig. 5, taken after readmission of oxygen,

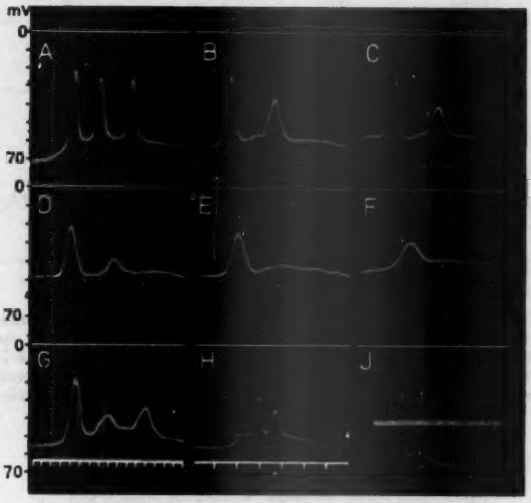

Fig. 7. Asphyxial and postasphyxial changes in interneuron activated by single shock stimulation of dorsal root. *A* repetitive response before asphyxia; *B* 60 sec, *C* 70 sec, *D* 79 sec, *E* 90 sec and *F* 145 sec after stop of respiration; *G* 5 min, *H* 5 min 15 sec and *J* 5 min 25 sec after restoration of ventilation. Time in msec in *G* valid also for *A*—*F*. Time in 5 msec for *H* and *J*.

show that in this case a pronounced postasphyxial hyperpolarization develops (cf. also Fig. 6). Of special interest is the fact that the prepotential — with unchanged stimulus strength — has increased in amplitude, although not sufficiently to initiate an impulse.

II. Asphyxial changes in interneurons.

Interneurons form a heterogeneous group of cells both from an anatomical and a physiological point of view (cf., *e. g.*, KOLMODIN 1957), and different reactions to asphyxia can *a priori* be expected. Our experience of asphyxiation of interneurons is as yet limited but some examples from the experiments made so far will be presented.

In Fig. 7, record *A* shows the characteristic repetitive response of a dorsal horn interneuron to suprathreshold afferent stimulation, obtained just before respiration was stopped. Records *B*—*F*

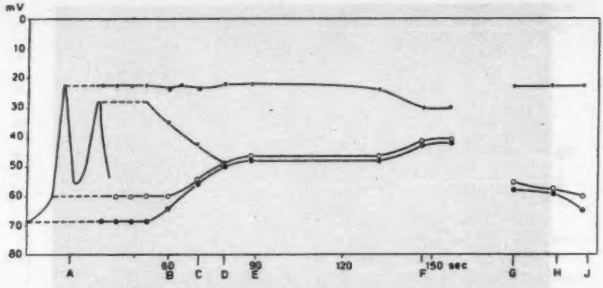

Fig. 8. Graphical representation of successive changes in membrane potential level (*large filled circles*), firing level (*open circles*) and spike peak level (*small filled circles*) of interneuron during and after a 180-sec period of asphyxiation. Same experiment as Fig. 7 (cf. letters on time scale). Time in sec from stop of respiration. *G* 5 min, *H* 5 min 15 sec and *J* 5 min 25 sec after restoration of ventilation.

show the changes during a 2.5-minute period of asphyxia. The successive changes in membrane potential, firing level and spike peak level of the first spike can best be followed in the diagram Fig. 8. The membrane potential level decreased, in a first stage, from the initial value of 70 mV to 50 mV and, in a second stage, to nearly 40 mV. With the decrease in membrane potential the prepotential becomes successively smaller, as was the case for the motoneuron in Fig. 5.

The spike potential behaves in the same way as the motoneuron spike also in so far as there are only insignificant changes in spike peak level until the late phase of depolarization. A definite change in the shape of the first spike can be seen quite early, both rising and falling phases being considerably slower than normal even in *B*. Derivation of these potentials performed by the same method as in Fig. 4 showed that in record *C* the maximum rate of rise of the ascending spike phase as well as the maximum rate of decay of the falling phase had decreased by about 50 per cent. No indication of any notch on the rising phase was found in this case (cf. HAAPANEN, KOLMODIN and SKOGLUND 1958).

The second and third spikes show similar changes as the first one but are more sensitive to asphyxia and have both completely dropped out at a time when there is only a small change in the peak level of the first spike (*E*). The successively prolonged intervals between the spikes should also be noticed. A recovery back

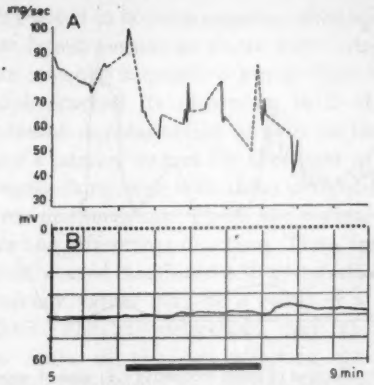

Fig. 9. Changes of spike frequency (*A*) and of membrane potential level (*B*) of naturally discharging interneuron during a 110-sec period of asphyxiation (*thick black line*). *Dotted lines* in *A* correspond to periods when no spike recording was made. Time in minutes after impalement. Time between vertical lines 30 sec. See text.

to the initial state was obtained within little more than 5 minutes of reventilation, as appears from records *G*—*J* (Fig. 7) and from the diagram Fig. 8.

Since normally many spinal interneurons are in a state of rhythmical firing (cf. KOLMODIN and SKOGLUND 1954, FRANK and FUORTES 1956) it was of a certain interest to study how asphyxia affected this type of activity. We have chosen two examples of asphyxial reactions of interneurons which maintained such a continuous firing.

In the experiment Fig. 9 the interneuron was exposed to a 110-sec period of asphyxia. As appears from *B* there are small fluctuations in the membrane potential level after 30 sec but a more significant depolarization did not appear until after the end of the asphyxial period. This cell showed the large fluctuations in its normal discharge frequency that may be characteristic of interneurons (cf. KOLMODIN 1957) but the changes induced by asphyxiation are still obvious. Before the asphyxia the discharge frequency (*A*) varied between 75 and 100 per sec; during asphyxiation there was an obvious tendency to a lowering of the frequency which at the end of the period of arrested res-

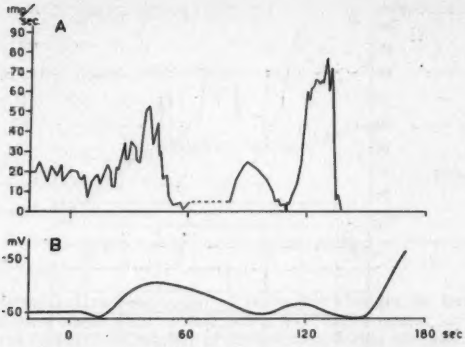

Fig. 10. Changes of spike frequency (*A*) and of membrane potential level (*B*) of naturally discharging interneuron during a 180-sec period of asphyxia. *Dotted line* in *A* as in Fig. 9. Time in sec from stop of respiration. See text.

piration varied between 50 and 85 per sec; immediately after the asphyxia, corresponding to the period of increased depolarization in *B*, there was a further lowering of the discharge frequency.

The next example (Fig. 10) shows more pronounced effects of the same kind; in this case the asphyxial period was maintained until complete block was attained. Initially this cell was discharging at a slow, rather constant rhythm of about 20 per sec (*A*). After 20 sec of asphyxia there was an increase in frequency followed by a brief period of block. After about 80 sec the cell resumed its initial rhythm for a short period, after which there was a burst of high-frequency activity, finally changing into definite block. The membrane potential level (*B*) showed fluctuations in depolarizing direction after 20 sec of asphyxia; simultaneously with the definite block of impulse discharge there was a dramatic lowering of the membrane potential.

Discussion.

By means of a needle electrode inserted in the cord and another on a ventral root VAN HARREVELD (1946) measured the potential differences between the grey matter of the cord and the anterior root during asphyxiation of the cat's spinal cord. He found that

the grey matter started to become negative after about 40—60 sec, while such short latent periods as 20 sec were obtained only after feeding nitrogen into the respiration pump. This initial phase of negativity, which reached its maximum in 2—4 minutes, was thought to represent depolarization of cells in the grey matter. VAN HARREVELD's latency values for the onset of the asphyxial depolarization agree fairly well with those obtained in the present investigations on motoneurons. There are several factors which may account for the differences observed. Thus, for instance, VAN HARREVELD mostly used nembutalized preparations. Our somewhat smaller latency values may be a result of a more sensitive recording method. The microelectrode may also have caused changes in the state of the cell affecting its sensitivity to metabolic changes. This might be one explanation of the incomplete recovery of the membrane potential of the investigated cell often found after asphyxia at a time when a neighbouring cell shows full membrane potential on penetration.

The above data of the membrane potential values must be seen against the background that the absolute value of the membrane potential level, in moto- as in interneurons, cannot be determined with full certainty (cf. HAAPANEN, KOLMODIN and SKOGLUND 1958); in the present asphyxia experiments dislocation of the cord tissue relative to the electrode due to minute movements of the animal or to blood pressure changes might also have induced artefact components in the potential changes recorded.

The predominant part of the changes in membrane potential level observed, *e. g.*, in motoneurons is most likely a direct effect of asphyxiation on the membrane of the cell recorded from. It should be emphasized, however, that some part of the variations may be secondary, caused by changes in the background activity of the interneurons influencing the motoneuron; pronounced variations in the rhythmical interneuron activity have been demonstrated even at an early stage of asphyxia, and the initial changes in membrane potential — whether decrease or increase (cf. Fig. 6) — might therefore be ascribed to such variations.

In this connection it is of interest to note that KIRSTEIN (1951) found an early depression of the monosynaptic reflex during asphyxiation experiments on unanesthetized spinal preparations, which was abolished after administration of dial. Since this drug is known to depress interneuron activity he drew the conclusion that the depressive phase was due to increased internuncial ac-

tivity acting on the monosynaptic pathway. This interpretation is strongly supported by our finding that such periods of increased rhythmical activity may actually occur in the early period of asphyxia.

LLOYD (1953) studied the influence of asphyxia on antidromically activated motoneurons in the cat's spinal cord, by recording from the ventral root or cord surface, and was able to draw fundamental conclusions about the membrane potential changes in the intramedullary parts of the motoneuron. He described a first stage in which there was a loss of somatic afterpotential and concluded that this was connected with central depolarization. These changes took place after about 60 sec and were completed in about 90 sec. After a period of fluctuations of somatic responsitivity accompanied by intermittent impulse discharges a complete block followed after 3—4.5 minutes. The block was assumed to be of cathodal character and to take place between the initial and the myelinated segment of the motor axon. With intracellular recording, the slow spike repolarization processes were also affected at an early stage, simultaneously with the decrease in membrane potential level (cf. Fig. 2). However, before these results can be fully interpreted it is necessary further to analyse the complex relation between the membrane potential level and the spike repolarization in spinal neurons (cf. KOLMODIN and SKOGLUND 1958). In our experiments with antidromic stimulation the final block appeared during the late phase of pronounced depolarization, but we cannot from the present evidence draw any conclusions about its localization. Fluctuations in membrane potentials and spontaneous firing of the motoneurons have also been observed by us within the time period indicated by LLOYD.

From changes in electrotonic ventral root potentials LLOYD drew the conclusion that the motoneuron (in analogy with peripheral nerve, GERARD 1930, LORENTE DE NÒ 1947) displays a phase of postanoxic repolarization overshooting the normal level which leads to a block of anodal type, probably located in the soma. The intracellular recording technique has now provided direct evidence that membrane potential values exceeding the preasphyxial level may be attained following readmission of oxygen.

We found that with orthodromic activation the spike was blocked at an earlier stage of the asphyxia than with antidromic stimulation, which is in accordance with observations by BROOKS and ECCLES (1947) in experiments with focal recording. The dif-

ferent pre- and postsynaptic processes which may be involved in the blocking of the orthodromic spike have not been a subject for analysis in the present investigation.

Before the final block occurs both the anti- and orthodromic motoneuron spikes undergo characteristic changes in configuration, and similar changes could also be demonstrated in interneurons. The definite decrease in maximum rate of rise of the ascending, as well as in the rate of decay of the descending, spike phases, which was revealed by derivation, indicates a slowing-down of the underlying membrane processes. In principle, this result is in accordance with the findings on asphyxiated peripheral nerve in which a prolongation of the spike potential was early demonstrated (GERARD 1930). Basically similar events may occur in both types of membranes, but the more complex building-up of the motoneuron spike has to be taken into account (cf., *e. g.*, ECCLES 1957). The derivation curves of Fig. 4 indicate a differential behaviour of the two phases of the rising limb of the spike during asphyxia, but the inexact method used for reproduction of the potentials (cf. above) does not permit any definite conclusions to be drawn. It is obvious, however, that derivation performed simultaneously with the recording of potentials during asphyxiation experiments might provide valuable information.

GELFAN and TARLOV (1955) have reviewed earlier data on the different susceptibility of different types of cord cells to asphyxia. From their own investigations, using spinal cord potentials as index, they came to the conclusion that motoneurons were blocked approximately 1.5 times faster than interneurons. It is also our experience from the present limited series of experiments that interneurons are more resistant to asphyxia than motoneurons in so far as the changes in their membrane potential level are less marked during similar periods of asphyxia, as is evident for instance from a comparison of Figs. 1 and 8. Conclusive evidence on this point requires further investigations, several sources of error having to be taken into account. Thus, a comparison of the results from one preparation to the other may be misleading owing to variations in the state of the preparations with respect to oxygen saturation. On the other hand, comparative experiments of different types of cells in one and the same preparation may be complicated by the changes induced by repeated periods of asphyxia. Besides, the difference in absolute values of the

initially recorded membrane potential levels of moto- and interneurons makes a direct comparison difficult (cf. HAAPANEN, KOLMODIN and SKOGLUND 1958). Considering the well established difference between various types of peripheral nerve fibres to anoxia it is, however, not unlikely that the difference observed between the two types of cells is significant, indicating actual differences in membrane properties.

It is evident that many of the results obtained in the present investigation can be correlated to earlier studies on cord asphyxia, but the intracellular recording technique has also provided data about the asphyxial reactions of spinal neurons which could not be predicted from previous analyses. To these results belongs the finding that the successive diminution in total spike amplitude during asphyxia is the result of two different events: in the initial phase of asphyxia a reduction of the spike height takes place largely as a result of a lowering of the membrane potential level, whereas during the later phase there occurs, in addition, a drop in spike peak level. This result is of a certain interest in view of LORENTE DE NÓ's (1947) demonstration of a two-phase action of anoxia on frog nerve membrane: a first phase of decrease of the slow L-fraction of the membrane potential followed by a reduction of the Q-fraction, associated with the spike potential; the postanoxic hyperpolarization on the other hand was ascribed to an increased L-fraction. As discussed in a previous paper (KOLMODIN and SKOGLUND 1958) membrane processes in structures as different as frog nerve and the soma of mammalian spinal neurons cannot be directly compared, and too far-reaching conclusions should therefore not be drawn from the analogies between the two series of experiments. It seems to us, however, that asphyxia — or preferably anoxia under control of O_2 and CO_2 tension — should provide a useful tool in the further analysis of the basic membrane processes of spinal neurons.

Summary.

1. By means of intracellular recording the influence of brief periods of asphyxia on spinal moto- and interneurons has been studied in decapitate, curarized cats, the artificial ventilation of which was suspended for periods from 1 to 3 minutes.

2. In motoneurons the first change in membrane potential level

was usually observed after about 30—40 sec in the form of a moderate depolarization of 5—10 mV, lasting for at least 30 sec, after which follows a phase of rapidly developing depolarization which, if maintained for a sufficient time, becomes irreversible. During the recovery phase, which shows great variations, the motoneuron may pass through a stage of increased polarization.

3. Antidromic motoneuron spikes undergo a successive diminution in amplitude, at first owing to the decrease in membrane potential level, later as a result also of a change of the spike peak level. During this later phase, which corresponds to the pronounced fall in membrane resting potential level, a decrease both of the rate of rise of the ascending and the rate of decay of the descending spike phases could be demonstrated. Orthodromic motoneuron spikes showed similar changes but were often blocked at an earlier stage of asphyxia.

4. In interneurons changes in membrane potential levels similar to those in motoneurons could be demonstrated, but on the whole interneurons seem to be less susceptible to asphyxia. Orthodromic interneuron spikes set up by afferent single shock stimulation undergo essentially the same changes as described for motoneuron spikes.

5. Naturally occurring rhythmical interneuron discharges are affected in a characteristic way, showing periods of increased and decreased frequency, before final blocking — associated with a more pronounced decrease of the membrane potential level — occurs.

6. The results have been discussed in relation to earlier findings from experiments on spinal cord asphyxiation as well as on anoxia of peripheral nerve.

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From Johan Throne Holst's Institute for Nutrition Research, University of Oslo, Norway.

A Rapid Method for the Study of the Effect of Unsaturated Fatty Acids on Cholesterol Metabolism in Rats.

By

JENS G. HAUGE and RAGNAR NICOLAYSEN.

Received 21 July 1958.

Abstract.

HAUGE, J. G. and R. NICOLAYSEN. A rapid method for the study of the effect of unsaturated fatty acids on cholesterol metabolism in rats. *Acta physiol. scand.* 1959. *45*. 19—25. — A method for biological testing of the effect of unsaturated fatty acids on the cholesterol metabolism in rats is described. Pregnant mothers and the litters born are given a diet containing 10 % hydrogenated coconut oil and 1 % cholesterol. The young rats grow approximately at a normal rate the first 4 weeks of life; in the following 50 days weight increase slows down and very high liver and plasma cholesterol values result together with various symptoms of essential fatty acid deficiency. Serum cholesterol is analysed in each rat, and grouping follows according to sex and serum cholesterol values so that equal group values result for zero day. Next the fatty acids or fats to be tested are dosed in geometrically increasing doses with 10—12 rats in each group. Following two weeks daily dosing, a new blood sample is collected and the depression of serum cholesterol for each group is calculated. Liver and plasma cholesterol are approximately equally depressed, and group reductions of more than 50 % have been observed.

The correlation between serum cholesterol and cardiovascular disease is in the focus of interest in present day medicine. Many doubt the causal relationship, and the crucial experiment is still missing. However, work continues along a variety of lines, and the influence of the amount and type of fat in the diet on the serum cholesterol concentration is the subject of intensive research both in man and animals [*Man, i. a.* GROEN *et al.* (1952), KINSELL *et al.* (1953), MALMROS and WIGAND (1955), BEVERIDGE, CONNEL and MAYER (1956), BRONTE-STEWART, EALES and BROCK (1956), KEYS, ANDERSON and GRANDE (1957); *Animals, i. a.* SWELL *et al.* (1955), SHAPIRO and FREEDMAN (1955), KRITCHEVSKY *et al.* (1954)].

It is well established that poly-unsaturated fatty acids reduce serum cholesterol in humans, whereas saturated fatty acids have an adverse effect. In the majority of observations in animals, corresponding results have been achieved. In view of the fact that mass experiments are readily carried out in small laboratory animals, and because rigid standard conditions can be established in animals, it was thought that it would be of value to develop a method by which effects could be measured. PIHL (1954) in this laboratory, studied the regression of cholesterol fatty livers in rats. It was found that this is rapid when the *causa movens* (cholesterol and fat load in the diet) was eliminated.

In a number of pilot experiments conducted in the course of the last two years, it was observed that the regression of liver and serum cholesterol in rats given a diet containing 10 per cent hydrogenated coconut oil and 1 per cent cholesterol, was deeply influenced by the supplementation of small amounts of poly-unsaturated fatty acids. In the course of the experiments it became clear that work with total serum cholesterol would give the method aimed for.

The development of the method is described below. The succeeding paper gives the results obtained with some fatty acids and oils.

Experimental.

Chemical methods. Total liver lipids were determined according to FOLCH, LEES and SLOANE STANLEY (1957). Serum total cholesterol was analysed according to HAUGE and NICOLAYSEN (1958). The liver total cholesterol was analysed according to the same method, following saponification and extraction of the unsaponifiable matter (U.S.P. XV method). The values for normal rats out of our stock colony were:

total lipids about 4.5 %, serum cholesterol 60—80 mg % and liver total cholesterol about 0.3 %.

Biological. The aim of the work here presented was to develop a method which would allow simultaneous observations of effects on growth and on cholesterol regression.

In some preliminary work 1 per cent cholesterol in a fat free diet given to weanling rats from our stock colony, cessation of growth did not follow until 14 weeks of age. Next, we introduced a fully hydrogenated fat in the diet and used the procedure familiar from vitamin assays, giving the mothers the experimental diet early in pregnancy. The final diet consists of:

Casein (acid precipitated, commercial)	2,000	g
Hydrogenated coconut oil (iodine number of 2)	1,000	g
Sucrose	6,037	g
Salt mixture, HAWK, OSER no. 3)	400	g
Vitamin B mixture (GREENBERG *et al.* 1950)	26	g
Roughage (powdered wood)	400	g
Choline hydrochloride	12	g
Tocopherol acetate (in 25 g sucrose)	0.5	g
Cholesterol (Collet & Co, USP standard)	100	g

Vitamins A and D were given in water soluble form at daily doses of about 50 and 10 units respectively (Collett & Co, Vimax pellets).

From this procedure litters of 5—6 result, compared with 8—9 in our stock colony rats. In the first 4 weeks the rate of growth is scarcely reduced. In the following weeks male rats grow at the rate of about 2 g daily, *i. e.* about half the rate of our normal rats. Within 2—3 months, clear signs of essential fatty acid deficiency develop. Only in one series of experiments did growth stop at this age. In the later experiments growth continued slowly. The rats were taken into the experiments at the age of about 70 days. In some early experiments we tried vitamin free casein and the commercial casein used later on in parallel experiments. No difference in rate of growth or development of skin symptoms was found between the two groups.

Results.

Table I gives the result of an early pilot experiment in which 40 mg soya-bean oil was given daily by pipette for 8 and 12 days respectively.

It is apparent from the table that the soya-bean oil supplementation resulted in a more or less pronounced drop in all the variables. There was, however, considerable variability, a fact that has become even clearer from later experiments. In male rats, the serum cholesterol has mostly been in the range of 150 to 350 mg

Table I.

The effect of 40 mg soya-bean oil for 8 and 12 days on liver and serum cholesterol in rats with essential fatty acid deficiency.[1]

Number of animals	Group	Days of exp.	Body wt. g	Liver wt. in % of body wt.	Liver lip. %	Liver chol. %	Ser. chol. mg %
2	control	8	71	5.8	6.1	0.81	174
2	»	12	101	5.1	7.9	1.41	280
2	soya-bean	8	75	5.5	5.6	0.65	155
3	supplemented	12	88	5.1	6.2	0.71	150

[1] The mothers were given a fat free diet with 1 per cent cholesterol. From weaning, the experimental rats received the final experimental diet for 4 weeks.

per cent, in female rats in the range of 300 to 700 mg per cent, and the liver cholesterol lay in the range of 1 to 4 per cent. Both for liver and serum cholesterol some extreme values were also observed outside these ranges.

Under such conditions, the absence of any knowledge of the initial liver and serum cholesterol values is a serious handicap and makes comparison of the groups more difficult. Instead of comparing the cholesterol level in experimental groups following fatty acid supplementation with that of a control group, it was decided to measure cholesterol in each animal before and after treatment. The control group was retained in order to have an indication of the spontaneous variation in serum cholesterol.

Next it was of interest to study in some detail the correlation between liver and blood cholesterol indicated in Table I. For this purpose on zero day the left big lobe of the liver was removed from six female rats, and blood was drawn from their tails. Following 14 days of dosing with 80 mg daily of soya-bean oil the animals were killed and the rest of the liver and serum was analysed for cholesterol. Prior to this it had been observed that the left big lobe would give cholesterol values 9 ± 3 per cent above the rest of the liver.

The results are given in Table II. A control group with serum and weight data only from the same experiment is also included in the table.

A considerable decline is evident, both in liver and serum cholesterol for the supplemented group as a whole, the average percentage drop being 31 and 53 per cent respectively, when the

Table II.

The effect of 80 mg soya-bean oil daily on liver and serum cholesterol in individual rats.

Suppl.	Rat. no.	Serum cholesterol, mg % 0 day	14. day	% decrease	Liver cholesterol, % 0 day	14. day	% decrease	Wt. gain g
80 mg soya-bean oil daily ...	1	212	88	58	1.89	1.18	38	16
	2	361	321	11	3.43	2.23	35	19
	3	655	235	64	3.03	2.60	14	16
	4	249	68	72	2.38	1.20	50	14
	5	469	215	54	6.22	2.52	59	22
	6	602	218	64	3.33	1.82	45	18
	Average:	425	191	53±9[1]	3.38	1.92	40	18
Control group	7	331	249	25				7
	8	168	156	7				9
	9	397	439	—10				6
	10	815	977	—20				6
	11	983	499	49				9
	12	209	283	—35				8
	Average:	483	433	3±13[1]				8

[1] S. E. of mean.

above mentioned difference of cholesterol distribution within the liver has been allowed for. The correlation between reduction in liver and serum cholesterol is not highly significant. The table furthermore gives an impression of the variation in the individual response to supplementation with unsaturated fats. It has been frequently observed (see HAUGE and NICOLAYSEN 1959) that this variability is relatively small at high dose levels, but considerable at low levels and particularly in the control groups.

In separate experiments the serum cholesterol was analysed for 14 days at intervals. In unsupplemented rats it was observed to vary irregularly. In rats insupplemented with unsaturated fats the fall was steady when high doses (80—100 mg daily) was given. In some instances serum cholesterol was even normalized in the course of 14 days, *e. g.* in rats no. 1 and 4 in Table II. The supplementation also resulted in a distinct increase in the rate of growth. The period of 14 days seemed to be too short to allow evaluation of the course of the skin symptoms.

In all preliminary studies male and female rats were tested separately with appropriate control groups. This separation, however, is not essential, and the method in its final elaboration, as employed in the accompanying paper, utilizes mixed groups.

Another advantage of the method lies in the distribution of the individual animals within the experimental groups according to serum cholesterol values at zero day. The final procedure in addition to the proper distribution according to age and litter establishes the groups with about the same number of the two sexes and with approximately the same average serum cholesterol.

Summary.

A method has been developed for the simultaneous production of essential fatty acid deficiency and hypercholesterolaemia in young rats. Pregnant rats receive a 1 per cent cholesterol diet containing 10 per cent hydrogenated coconut oil as the only fat. Growth retardation, skin symptoms and a large increase in serum and liver cholesterol develop within 2 to 3 months from birth. At this stage the rats are divided into groups receiving daily for 14 days 20 to 100 mg polyenoic acids or fats rich in these acids. The total serum cholesterol is analyzed in each rat at the start of this regimen, and again after 14 days. Control groups are run in parallel. The supplementation frequently results in reductions in the serum cholesterol concentration of up to 50 per cent or more. In some rats the serum cholesterol may even become normalized. With the aid of partial hepatectomy at zero day, a substantial reduction of total liver cholesterol was also seen to follow the administration of polyenoic acid.

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The Serum Cholesterol Depressive Effect of Linoleic, Linolenic Acids and of Cod Liver Oil in Experimental Hypercholesterolaemic Rats.

By

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Abstract.

HAUGE, J. G. and R. NICOLAYSEN. The serum cholesterol depressive effect of linoleic, linolenic acids and of cod liver oil in experimental hypercholesterolaemic rats. *Acta physiol. scand.* 1959. *45.* 26—30. — With the aid of the method of HAUGE and NICOLAYSEN the serum cholesterol depressive effect of various samples of highly unsaturated fatty acids or oils was studied. Linolenic acid had a stronger depressive effect than linoleic acid, and the polyunsaturated fatty acids of cod liver oil seemed to have an even stronger effect, since the effect of whole cod liver oil was at least equal to that of linolenic acid.

With the aid of the method described in the preceding paper (HAUGE and NICOLAYSEN 1959) some tests were performed with linoleic and linolenic acids (purchased from The Hormel Foundation), with a cod liver oil and a Furfurol extract of the fatty acids from the same oil (Fishery Research Institute, Bergen).

Results.

The results with linolenate, linoleate, cod liver oil, and a fraction of cod liver oil are presented in the table. It is evident that small amounts of these polyunsaturated methyl esters and fats will depress serum total cholesterol strikingly in spite of a daily load of about 1,000 mg hydrogenated fat and 100 mg of cholesterol. The best substantiated observation is that the polyenoic fatty acids of marine origin exert a profound depressive effect. The effects of pure di- and trienoic acids of plant origin seem also to be clear, and the results here presented indicate that linolenic acid has a stronger depressive effect than linoleic.

However, in the planning of the experiment based on some preliminary trials, we had anticipated somewhat different relative potencies of the di- and trienoic C_{18}-acids. The evidence for the considerably larger effect of linolenic acid as compared to linoleic in the present experiment should thus be taken with some caution until further results are available. On the other hand, it should be observed that the results with these acids are in line with the conclusions so far reached by AHRENS JR. *et al.* (1957) and by KEYS *et al.* (1957), namely that the depressive effect of unsaturated fatty acids is a function of the number of the double bonds in the acids.

The table lists the body weight gains in average for series I and II. One notes in several instances more than a doubling of the weight increase rate as a result of the administration of the oils and fatty acid metyl esters.

Discussion.

The small amount of cod liver oil or linolenate (40 mg per day in 14 days) that is required for maximal depression might suggest a catalytic function. This may very well be the case, through a restoration of enzymatic lipoproteins involved in cholesterol metabolism. There is, however, probably also enough fatty acids supplied to fulfill the hypothetical role of a transport agent for excess liver and blood cholesterol. A 33 per cent reduction in liver cholesterol and a 50 per cent reduction in serum cholesterol would for 5 g of liver and 5 ml serum with initial cholesterol concentrations of respectively 3 per cent and 400 mg per cent

Table I.

The change in serum total cholesterol following two weeks daily administration of polyunsaturated fatty acids.

(Diet: 10 per cent hydrogenated coco-nut oil, 1 per cent cholesterol.)

Series	Type of fatty acid	Daily dose mg	Depression of serum cholesterol (per cent of zero day value[2])	Average serum cholesterol at zero day mg %	Increase in body wt in 14 days	No. of rats	
						f.	m.
I	Linoleic	20	— 7 ± 8[1]	312	26 ± 2[1]	5	7
		40	16 ± 9	313	28 ± 4	5	7
		80	35 ± 5	298	37 ± 4	5	7
	Linolenic	40	46 ± 5	331	32 ± 2	4	6
		100	51 ± 3	307	36 ± 3	5	7
	Cod liver oil[3]	20	33 ± 7	311	24 ± 2	6	6
		40	49 ± 8	321	34 ± 2	5	6
		80	52 ± 5	336	34 ± 3	4	7
	Control group	0	6 ± 9	324	17 ± 2	7	7
II	Cod liver oil[3] fatty acids	20	33 ± 8	494	23 ± 2	6	
		40	59 ± 12	472	21 ± 2	6	
		80	65 ± 4	482	27 ± 3	6	
	Control group	0	— 3 ± 14	485	15 ± 3	6	

[1] S. e. of mean.

[2] Corrected for change in control group (per cent change in control group subtracted from change in test group).

[3] Supplied by The Fishery Research Institute, Bergen, which reports the following fatty acid composition (according to analyses (alkali isomerization method) by professor O. NOTEVARP, NTH, Trondheim):

Fatty acid	Cod liver oil fatty acids (Furfurol-extracted from the cod liver oil)	Cod liver oil
hexaenoic	13.3	14.0
pentaenoic	15.3	12.0
tetraenoic	4.9	3.0
trienoic	0.8	1.7
dienoic	2.7	4.2

correspond to only 60 mg cholesterol. For complete normalization another 150 mg would have to be removed. In addition we must, however, envisage a requirement of unsaturated acids for the transport and metabolism of the fraction absorbed of the daily load of 100 mg cholesterol.

Other workers (HEGSTED *et al.* 1958) have felt the need for rapid experimental small animal tests. With the aid of cholic acid and cholesterol in the diet they obtain in the course of some weeks very high serum cholesterol values. So far they have tested a number of fats with a considerable range of variety in the fatty acid composition. The evaluation of the material produced lead to the conclusion that the essential fatty acids (linoleic and perhaps arachidonic acid) act together with the saturated fatty acids in causing low serum cholesterol values. The unsaturated non-essential fatty acids (oleic, linolenic and eleostearic) apparently promoted high serum cholesterol values and this action was counteracted by the essential and saturated fatty acids. In fact the highest serum cholesterol values were obtained with triolein as the sole source of fat in the diet, and the addition of coco-nut oil was followed by a reduction of serum cholesterol.

The results here presented are not very numerous, however, the effect of small amounts of linolenic acid is very striking; and is at variance with the results of the Boston group. The effect of triolein is also at variance with the general conclusion of KEYS *et al.* (1951). The two rat methods are different in various respects, and this may explain the differences observed.

Summary.

With the aid of the rat method of HAUGE and NICOLAYSEN (1959) it was found that cod liver oil and a Furfurol fatty acid fraction of the same oil strikingly depressed serum cholesterol. Parallel tests with methyl esters of linoleic and linolenic acids (Hormel Foundation) also resulted in a considerable reduction of serum cholesterol. In this experiment linolenic acid had a stronger action than linoleic acid.

Addendum.

Recently the following additional results were achieved.

1. *Linoleic versus linolenic acid.* (Hormel Foundation methylesters.) The procedure was identical to that described in the above paper.

Daily dose of: (40 mg daily for 14 days)	Serum cholesterol		No. of rats	
	zero day value	per cent depression	f.	m.
Linoleic acid	414	21 ± 6	9	10
Linolenic acid	412	42 ± 5	9	11

2. In 8 rats at zero day the liver cholesterol averaged 4.4 percent. Following a daily dose of 80 mg for 14 days of the same cod liver oil as used in the above described experiments, the liver cholesterol was reduced to 2.1 %. The simultaneous serum cholesterol depression was 60 percent (zero day value 350 mg %).

Conclusion: These results substantiate the conclusion that linolenic acid exerts a stronger plasma cholesterol depressive effect than linoleic acid. The no. of double bonds seems to be a decisive factor under the present experimental conditions.

Financial support received from New York Community Trust.

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Influence of Dietary Cholesterol, Cod Liver Oil and Linseed Oil on Cholesterol and Polyenoic Fatty Acids in Tissues from Fasted and Non-Fasted Chicks.

By

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Abstract.

DAM, HENRIK, GUNHILD KRISTENSEN, GUNHILD KOFOED NIELSEN and EBBE SØNDERGAARD. Influence of dietary cholesterol, cod liver oil and linseed oil on cholesterol and polyenoic fatty acids in tissues from fasted and non-fasted chicks. *Acta physiol. scand.* 1959. *45*. 31—42. — The deposition of cholesterol and polyenoic fatty acids after feeding diets with no fat, 10 % cod liver oil or 10 % linseed oil with or without 1 % dietary cholesterol for a 4 week period was determined in various tissues of chicks. About half of the chicks examined had no access to food during the last 16 or 18 hours before they were killed. Data for cholesterol in plasma, aorta, heart, liver, brain and fat tissue and for polyenoic fatty acids in heart, liver, brain and fat are presented.

In previous investigations we have studied the influence of dietary cholesterol and unhydrogenated or hydrogenated peanut oil on the content of cholesterol and polyenoic fatty acids in tissues of chicks (DAM *et al.* 1956, 1958 a).

Table I.

Basal diet (g per kg).

Casein, crude[1]	200
Gelatine	80
Salt mixture no. 4[2]	51.7
Vitamin mixture no. 4[2]	1.0
Choline chloride	1.5
Sucrose	665.8
	1,000.0
d, l-α-tocopherol acetate[3]	100 mg
Vitamin K substitute[4]	10 mg

[1] "Dairinex", from A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark, containing 1.2 % fat. The fat contained 1,7 % dienoic, 0.7 % trienoic, and 0.1 % tetraenoic acid.

[2] DAM *et al.* 1958 b.

[3] Ephynal, "Roche", kindly furnished by the manufacturer.

[4] Dicalcium salt of 2-methyl-1, 4-naphthohydroquinone diphosphoric acid ester (Synkavit, "Roche"), kindly furnished by the manufacturer.

The present experiments were carried out in order to examine the influence of fats containing more highly unsaturated fatty acids than those occurring in peanut oil on the deposition of cholesterol in plasma, aorta, heart, liver, brain, and fat tissue, and on polyenoic fatty acids in the same tissues (except plasma and aorta).

A further object of the present experiment was to study the possible influence of fasting.

Experimental.

Sixty day-old chicks (male and female) received a commercial diet (DAM *et al.* 1957) for fourteen days. Thereafter they were distributed into 6 groups (10 chicks per group) and given the experimental diets. These diets contained no fat, 10 % cod liver oil (CLO), or 10 % linseed oil (LSO), respectively, and 0 or 1 % cholesterol. The fat component and the cholesterol were incorporated in the basal diet (Table 1) instead of the corresponding weight of sucrose. Vitamins A and D_3 were given in "aqueous" solution,[1] 0.1 ml twice a week per animal, corresponding to 250 I. U. A and 20 I. U. D_3 per day.

[1] Vitamin A acetate ("Roche") 1 g; crystalline vitamin D_3 ("Roche") 0.0058 g; ethyl alcohol, 100 ml; "Tween 80" 64 g; and distilled water ad 330 ml.

Cod liver oil and linseed oil were analyzed for polyenoic fatty acids by isomerization according to the method of HAMMOND and LUNDBERG (1953) with the following results:

Cod liver oil (CLO) (calculated for pentaene C_{20}[1])	Linseed oil (LSO) (calculated for pentaene C_{22})
2.4 % dienoic	11.7 % dienoic
0.0 % trienoic	50.6 % trienoic
5.1 % tetraenoic	0.2 % tetraenoic
9.8 % pentaenoic	0.0 % pentaenoic
12.0 % hexaenoic	0.6 % hexaenoic
total 29.3 % polyenoic	total 63.1 % polyenoic
+ 0.6 % conjugated dienoic	+ 0.4 % conjugated dienoic

Otherwise the experimental conditions and methods were as reported earlier (DAM *et al.* 1956, 1958 b), except that half of the chicks in each group were fasted during the last 16 to 18 hours before decapitation.

Results and Discussion.

Cholesterol in plasma and tissues (Table II).

The fasting period of 16 to 18 hours influenced the plasma cholesterol content of the chicks *on the fat-free diet without cholesterol*, the average value for the fasted chicks being significantly higher (about 50 %) than the average value for the non-fasted chicks.

The standard error of the mean of the plasma cholesterol values was lower for the fasted than for the non-fasted chicks when the diet contained 1 % cholesterol.

In the tissues there was no significant difference between the cholesterol values from fasted and from non-fasted chicks.

Feeding of the two fats without dietary cholesterol showed no significant differences with respect to cholesterol deposition in plasma and the tissues examined. In the case of plasma, the groups fed CLO and LSO, respectively, had the same average cholesterol values as the non-fasted fat-free group.

Feeding of the two fats caused a decrease in liver cholesterol concentration compared with the feeding of the fat-free diet. The same effect was found in an earlier experiment (DAM *et al.* 1956) with peanut oil.

Considering the individual cholesterol values from the fat-free

[1] KLENK and EBERHAGEN (1957) isolated eicosapentaenoic acid from cod liver oil.

Table

Cholesterol content expressed as mg % of plasma and

Group no.	1766		1767	
Addition to diet: fat	0		0	
cholesterol .	0		1 %	
	A fasted	B non-fasted	A fasted	B non-fasted
Plasma	$198^5 \pm 6$	$133^5 \pm 7$	$218^5 \pm 21$	$207^5 \pm 37$
Aorta (dry weight)	$609^5 \pm 42$	$639^5 \pm 4$	$850^4 \pm 71$	$928^5 \pm 146$
Heart	212^5	187^4	275^5	216^5
Liver	$410^5 \pm 19$	$453^5 \pm 52$	$826^5 \pm 161$*	$588^5 \pm 108$**
Brain	$1{,}098^4$	$1{,}154^4$	$1{,}253^4$	$1{,}178^4$
Fat tissue	77^5	67^5	110^5	92^5

* $668^4 \pm 37$
** $484^4 \pm 38$
} if values deviating considerably from the average are exluded.

The superscripts indicate the number of organs examined.

The cholesterol content of plasma, aorta and liver was determined for each five fasted and those from the five non-fasted chicks were pooled for analysis.

chicks on cholesterol-free diet (group 1766, Table III), it is seen that for non-fasted chicks lower plasma cholesterol values correspond to higher liver cholesterol values and vice versa. For fasted chicks in group 1766, higher plasma cholesterol values correspond to higher liver cholesterol values.

With 1 % dietary cholesterol, LSO caused higher cholesterol values for plasma, heart, liver, and fat tissue than did CLO. In this respect the effect of LSO resembles that of peanut oil (DAM *et al.* 1956).

Brain cholesterol always shows constant values regardless of the dietary regimen.

LSO and CLO gave nearly the same values for aorta cholesterol.

The degree of unsaturation, as expressed by the iodine values, is nearly the same for the two fats, but LSO contains mainly trienoic and some dienoic acid, whereas CLO mostly contains the more highly unsaturated fatty acids (tetra-, penta- and hexaenoic).

II.

mg per 100 g of tissues (wet weight, except for aorta).

1768		1769		1770		1771	
10 % CLO 0		10 % CLO 1 %		10 % LSO 0		10 % LSO 1 %	
A fasted	B non-fasted	A fasted	B non-fasted	A fasted	B non-fasted	A fasted	B non-fasted
135[5] ±12	131[5] ± 8	252[5] ± 14	286[5] ± 25	138[5] ± 9	137[5] ± 7	408[4] ± 71	570[5] ±167
662[5] ±22	590[5] ±58	1,252[5] ±123	1,438[5] ±171	587[4] ±39	591[5] ±22	1,335[4] ±186	1,440[5] ±110
184[5]	166[5]	250[5]	236[5]	191[5]	161[5]	356[4]	348[5]
321[5] ±17	280[5] ±16	1,751[5] ± 595†	1,393[5] ± 200†	308[5] ±10	263[5] ± 7	2,516[4] ±526	2,276[5] ±197
1,188[4]	1,207[4]	1,201[4]	1,128[4]	1,221[4]	1,126[4]	1,170[3]	1,262[4]
74[5]	66[5]	118[5]	88[5]	135[5]	75[5]	160[4]	99[5]

† 1,177[4] ± 205 / † 1,548[4] ± 134 } if values deviating considerably from the average are excluded.

chick individually. For heart and fat tissue, respectively, the organs from the Brains were pooled two and two for analysis.

Since feeding of 1 % cholesterol with 10 % CLO caused lower cholesterol accumulation in the organs examined (except brain and aorta) than feeding of 1 % cholesterol with 10 % LSO, it is likely that cholesterol is more easily metabolized in chicks when fed together with fat containing the more highly unsaturated fatty acids present in cod liver oil.

Polyenoic fatty acids in tissues (Table IV).

The *fat-free diet* causes accumulation of trienoic fatty acid in heart, liver and brain, as reported earlier (Dam *et al.* 1956, see also Dam *et al.* 1958 b), but not in fat tissue where polyenoic fatty acids are nearly absent when the diet is fat-free.

Dietary cholesterol without dietary fat does not seem to influence the deposition of polyenoic fatty acids. The fasted chicks show higher values for the individual polyenoic fatty acids in the

Table III.

Individual cholesterol values in plasma and liver from the fasted and the non-fasted chicks on fat-free, cholesterol-free diet (group 1766).

	Chick no.	Plasma mg %	Liver mg per 100 g
A, fasted	5495	184	387
	5496	185	348
	5499	206	437
	5502	207	445
	5503	210	434
B, non-fasted	5494	152	334
	5497	110	632
	5498	141	394
	5500	135	400
	5501	125	504

liver than do the non-fasted. This may be due to catabolism of saturated fatty acids and oleic acid during fasting whereby the percentage of polyenoic fatty acids in the total fatty acids becomes higher.

When the set of formulae valid for pentaenoic C_{20} are used for the calculation, CLO gives a rather varying distribution of polyenoic fatty acids in the organs examined. The amount of dienoic acid is highest in the heart, but not higher than for the corresponding fat-free group. In the fat tissue there is also some dienoic acid, but in the liver it is nearly absent, and for the brains negative values appear. The contents of trienoic acid show negative values except in the case of fat tissue, where small positive values are obtained.

These negative values for dienoic and trienoic acids may be due to 1) Standard error, which probably is about 0.2 %, or 2) Subtraction of (intermediate) conjugated compounds produced during the saponification procedure. Some of these conjugated compounds originate from the pentaenes or hexaenes. During the saponification, two or three of the five or six isolated double bonds may conjugate and are measured as preformed conjugated dienoic or trienoic and incorrectly subtracted from the total conjugated dienoic and trienoic formed by the isomerization procedure.

The values for tetraenoic acids in heart, liver, and brain are nearly the same as those obtained with the fat-free diet, but in fat tissue a small increase is observed.

The more highly unsaturated fatty acids from CLO are deposited in the organs according to different patterns. In heart, pentaenoic acid is found in the greatest amount, whereas hexaenoic acid is predominant in liver and brain. The fat tissue contains only small amounts of tetra-, penta-, and hexaenoic acids.

It appears as if cholesterol decreases liver hexaenoic acid. The influence of fasting is not significant.

Experiments by RIECKEHOFF, HOLMAN and BURR (1949) with CLO given to rats point in the same direction as the results obtained in the present study with chicks. These authors found marked deposition of pentaenoic and hexaenoic fatty acids in tissue lipids.

REISER (1950) studied the effect of feeding CLO to chicks and reported that the higher unsaturated fatty acids in CLO were converted to dienoic. However, his results were published before standards for pentaenoic and hexaenoic acids were available, wherefore only the extinction coefficients were given. Our results do not point to a conversion to dienoic but to deposition of more highly unsaturated fatty acids.

When the diet contains *10 % of LSO*, the most significant finding is an accumulation of pentaenoic fatty acid in heart and especially in the liver. Increase in pentaenoic acid is also observed in brain, but not in fat tissue.

In the linseed oil no pentaenoic acid is present, the deposition in organs must be due to conversion of other polyenoic fatty acids.

Only part of the large amount of trienoic acid in the LSO is deposited in the full extent; therefore it is probable that some of the linolenic acid has been converted to pentaenoic acid.

Dienoic acid is found in all tissues except brain.

Tetraenoic acid in the organs is nearly the same as for the fat-free groups, whereas hexaenoic is increased in liver and brain and to some extent in heart. The deposition is not very marked, wherefore it is probable that hexaenoic is derived from dienoic, not from trienoic acid.

These results are in contrast to those obtained by WIDMER and HOLMAN (1950) with rats fed pure ethyl linoleate and pure ethyl linolenate. They found that linolenic acid formed hexaenoic acid and some pentaenoic (especially in kidney and blood), and that linoleic acid did not raise the amount of hexaenoic.

Recently, STEINBERG *et al.* (1957) studied the metabolism of linolenic acid in rats with labelled methyl linolenate and found

Table

Polyenoic fatty acids in % of total fatty acids in tissues

Group no.		1766		1767	
Addition to diet: fat		0		0	
cholesterol		0		1 %	
		A fasted	B non-fasted	A fasted	B non-fasted
Heart[1]	% dienoic	6.4	6.1	5.9	6.1
	% trienoic	5.1	6.6	5.5	5.3
	% tetraenoic	3.9	5.0	3.2	3.8
	% pentaenoic	0.3	0.4	0.3	0.4
	% hexaenoic	0.0	0.1	0.2	0.0
	% preformed conjugated dienoic	0.5	0.7	0.4	0.5
	% total	16.2	18.9	15.5	16.1
Liver[2]	% dienoic	4.3	1.7	3.3	2.0
	% trienoic	7.6	5.1	8.5	6.1
	% tetraenoic	5.7	3.1	4.9	3.1
	% pentaenoic	1.2	0.6	0.9	0.5
	% hexaenoic	1.4	0.6	1.1	0.6
	% preformed conjugated dienoic	1.1	0.7	1.3	0.6
	% total	21.3	11.8	20.0	12.9
Brain[2]	% dienoic	0.1	−0.4	−0.3	−0.3
	% trienoic	5.9	5.7	5.7	6.0
	% tetraenoic	8.0	8.9	8.4	8.6
	% pentaenoic	1.9	2.5	1.5	2.4
	% hexaenoic	6.5	7.0	7.1	7.3
	% preformed conjugated dienoic	1.4	1.4	1.6	1.4
	% total	23.8	25.1	24.0	25.4
Fat tissue[1]	% dienoic	1.3	1.0	1.2	1.4
	% trienoic	0.3	0.2	0.2	0.1
	% tetraenoic	0.0	0.1	0.0	0.0
	% pentaenoic	0.0	0.0	0.0	0.1
	% hexaenoic	0.1	0.0	0.0	0.1
	% preformed conjugated dienoic	0.2	0.2	0.2	0.2
	% total	1.9	1.5	1.6	1.9

[1] Five organs were pooled. One determination was made for fasted and one
The analyses were carried out on individual organs.
Two organs were pooled. The figures are averages of two determinations.

IV.

of chicks after 4 weeks of experimental feeding.

1768		1769		1770		1771	
10 % CLO 0		10 % CLO 1 %		10 % LSO 0		10 % LSO 1 %	
A fasted	B non-fasted	A fasted	B non-fasted	A fasted	B non-fasted	A fasted	B non-fasted
5.7	3.9	5.5	4.5	20.7	15.3	16.0	16.7
—0.4	—0.1	—0.3	—0.3	11.1	11.3	14.0	13.5
5.5	4.4	6.0	5.4	5.7	5.2	3.6	4.4
7.4	6.7	7.4	8.0	6.1	8.1	4.8	6.3
5.6	5.9	4.9	6.1	1.1	1.8	0.7	0.8
1.5	1.5	1.2	1.0	0.9	1.2	0.8	0.7
24.9	22.3	24.7	24.7	45.6	42.9	39.9	42.4
0.6	0.4	1.7	—0.2	14.5	10.6	12.5	11.9
—0.6	—0.4	—0.5	—0.7	2.9	6.1	6.4	9.5
4.9	3.5	3.5	3.1	3.9	1.8	1.0	1.2
7.0	7.8	7.1	8.1	15.3	14.1	12.4	9.0
22.1	17.1	13.7	12.8	7.9	5.2	3.1	2.7
3.7	3.2	2.7	2.4	3.3	1.6	1.5	1.3
37.7	31.6	28.2	25.5	47.8	39.4	36.9	35.6
—2.0	—2.3	—1.7	—1.8	—0.7	—0.3	—0.7	—1.5
0.2	—0.4	—0.2	—0.4	—0.1	—0.4	0.3	0.0
7.2	7.3	6.4	6.9	7.3	7.2	7.7	7.5
2.3	1.8	2.2	2.1	4.3	3.2	4.4	4.1
16.1	17.0	16.2	16.5	14.3	13.3	14.5	15.2
2.7	3.0	1.8	2.2	1.9	2.1	2.1	2.4
26.5	26.4	24.7	25.5	27.0	25.1	28.3	27.7
4.1	3.5	2.8	3.0	10.2	9.2	10.1	10.1
0.5	0.3	0.5	0.5	23.9	26.6	24.9	27.9
2.2	2.2	1.9	3.0	0.7	0.5	0.3	0.4
2.7	2.9	2.0	1.9	0.2	0.4	0.4	0.4
2.3	2.4	1.8	2.6	0.6	0.2	0.2	0.3
0.6	0.5	0.5	0.6	0.9	0.6	0.6	0.7
12.4	11.8	9.5	11.6	36.5	37.5	36.5	39.8

for non-fasted chicks.

that linolenate was not converted to arachidonic but to eicosapentaenoic acid.

Studies of REISER (1950) concerning polyunsaturated fatty acids in chicks showed that dienoic acid was converted to pentaenoic, and possibly tetraenoic, and trienoic to tetra-, penta-, and hexaenoic acids.

REISER's results do not agree with those presented in this paper. However, the trienoic acid was given as a linseed product, which contained 24 % linoleate and 76 % linolenate. A part of the polyenoic acid formed may have originated from dienoic. Furthermore, REISER's values for pentaenoic and hexaenoic acids are given as the extinction coefficients because standards were not then available.

Cholesterol combined with LSO seems to give a little higher content of trienoic acid and a little lower content of the more unsaturated polyenoic fatty acids in heart and liver.

The difference between fasted and non-fasted LSO-fed chicks with respect to tissue polyenoic fatty acids is not significant.

Summary.

The deposition of cholesterol and polyenoic fatty acids after feeding diets with no fat, 10 % cod liver oil or 10 % linseed oil with or without 1 % dietary cholesterol for a 4 week period was determined in various tissues of chicks.

Cholesterol in plasma and tissues:

The fasted chicks on fat-free diet without cholesterol showed significantly higher plasma cholesterol than non-fasted chicks on the same diet.

In the tissues there was no significant difference between the cholesterol values from fasted and from non-fasted chicks.

Without dietary cholesterol there was no difference in tissue cholesterol between the cod liver oil fed and the linseed oil fed chicks.

With 1 % dietary cholesterol, linseed oil caused greater cholesterol values for plasma, heart, liver, and fat tissue than did cod liver oil.

Polyenoic fatty acids in tissues:

The fat-free diets caused a higher content of polyenoic fatty acids in the liver for the fasted chicks than for the non-fasted.

With all the other diets examined there was no significant difference between the fasted and the non-fasted groups.

Cod liver oil supplementation caused a marked deposition of pentaenoic acid in heart and liver and a smaller deposition of this acid in fat tissue and brain. Hexaenoic acid was very much increased in liver and brain, and to some extent in heart, but much less in fat tissue.

Addition of cholesterol to the cod liver oil diet did not seem to influence the deposition of polyenoic acids, except for a decrease of hexaenoic acid in the liver.

Linseed oil supplementation led to accumulation of pentaenoic acid in heart and especially in the liver, whereas the deposition of pentaenoic was smaller in brain and did not occur in fat tissue.

Trienoic acid decreased concurrently with the increase of pentaenoic acid in heart and liver.

Addition of cholesterol to the linseed oil diet caused slightly higher amounts of trienoic and slightly lower amounts of the more highly unsaturated fatty acids in liver and heart.

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From the Children's Clinic, University of Helsinki, Finland.

A Method for Continuous Recording of Movement.

By

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Abstract.

MÄKELÄ, P., E. I. WALLGREN and K. ÖSTERLUND. A method for continuous recording of movement. *Acta physiol. scand.* 1959. *45*. 43—47. — Mechanical methods have proved to be unsatisfactory for recording breathing movements in newborn. The authors have used for this purpose an electrometrical recorder, which is capable of recording movements on the basis of the changes of electrical capacitance. A few examples prove that the device is suitable for recording the pattern and frequency of various movements, *e. g.* pulse, breathing and the apex beat.

The recording of movement by mechanical means in biological research often lacks precision and involves numerous potential errors. For instance, slowness (inertia) and elasticity (compliance) lead to a certain inaccuracy, variable in degree, of recording. It is probable that electronics will make it possible to record movement without any interference and will obviate the disturbing factors mentioned.

For recording various movements such as respiration, pulse and apex beat, the authors adapted a method which has been widely

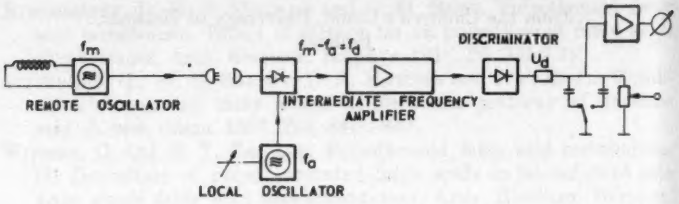

Fig. 1.

used for measuring purposes in technology. The recording device was constructed by Mr. J. Koivisto, M. Sc. (Eng.). It consists of the following components (Fig. 1):

1. Remote oscillator
2. Local oscillator and mixer
3. Intermediate frequency amplifier
4. Frequency discriminator
5. Tuning indicator

The frequency determining circuit of the remote oscillator has not been shielded and because of the stray capacitance of the circuit its frequency, f_m, is dependent on the distance to the conducting object (body) in the electric field (Fig. 2). This dependence is not linear but when the amplitude of the movement is relatively small compared with the distance, the frequency difference may be regarded with great accuracy as a linear function of the kinetic amplitude (Fig. 3).

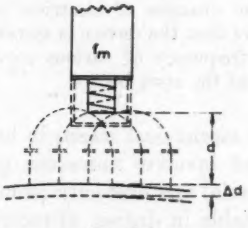

Fig. 2.

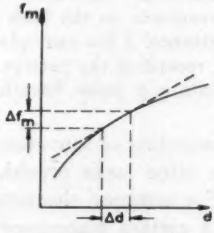

Fig. 3.

The difference frequency of the remote oscillator and the local oscillator, $f_m - f_a$, is fed to the amplifier which feeds the frequency discriminator. The output of the discriminator is a linear function of the difference frequency within its working range (Fig. 4).

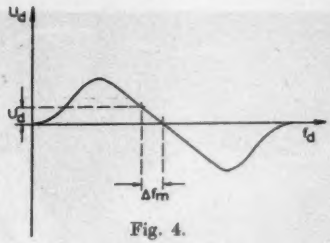

Fig. 4.

To tune the device ready for use, the frequency, f_a, of the local oscillator is adjusted until $f_m - f_a = f_d$ = the rated mean frequency of the frequency discriminator. The DC potential obtained from the discriminator is proportional to the frequency variation of the remote oscillator and thus to the distance difference between the coil of the remote oscillator and the object under study.

To assist the tuning of the device, the output of the discriminator is fed via an amplifier to the tuning meter in the front panel.

An adjustable attenuator and switch are coupled between the discriminator and output of the device. The switch makes it possible to choose the desired time constant to attenuate the disturbances.

The actual recording can be done by coupling the device to the ECG apparatus. The latter makes it possible to record simultaneously other functions, *e. g.* electrocardiogram, heart and breathing sounds. In their own experiments the authors usually employed Elema's direct recording Mingograf model 24 electrocardiograph.

Method: Fig. 5 illustrates the procedure itself. The most essential part of the equipment is the remote oscillator serving as a transducer, which can be easily introduced into the incubator by means of a flexible arm. The distance is governed primarily by the amplitude of the movement and the size of the object. The breathing of the newborn is best recorded at a mean distance of 2 cm. For a pulse rate recording the distance can be about 1 cm.

The device can thus be used, *e. g.* as in Fig. 5, to examine the breathing movement. A Maico amplifier was connected to the second channel of the ECG apparatus. The microphone made it possible to record the breathing sound simultaneously. Fig. 6 gives the result of the recording procedure. Fig. 7—8 give examples of other applications of the device.

Fig. 5. A remote oscillator which records the breathing movement of a newborn is placed about 2 cm from the thoracic wall. The breathing and the heart sounds are recorded simultaneously with a Maico electric stethoscope.

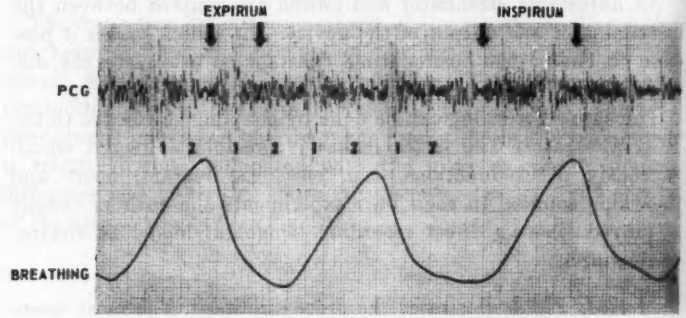

Fig. 6. Breathing sounds and simultaneous movements of the thoracic wall in a newborn. 1, first heart sound; 2, second heart sound.

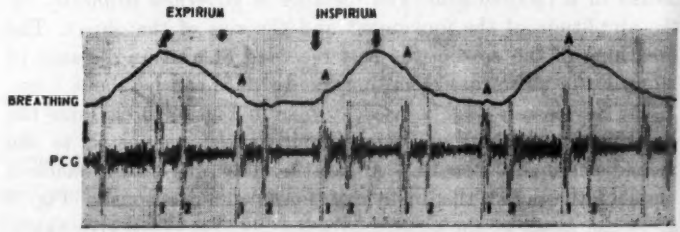

Fig. 7. Movement of the thoracic wall and breathing sounds in an adult. The apex beat, A, appears simultaneously with the first heart sound, 1, followed by the second heart sound, 2.

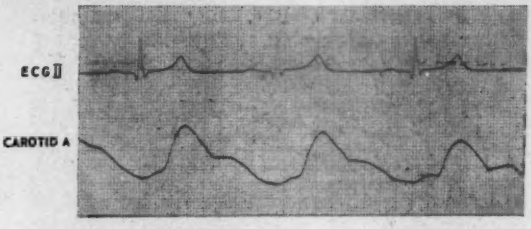

Fig. 8. Electrocardiogram and carotid artery pulse wave in a healthy adult.

Comments: The device is obviously suitable for recording the pattern and frequency of movements of different types. The most important advantages are the elimination of slowness, elasticity, friction and mass and thus, given certain conditions, the recording can be regarded as a fairly accurate projection of the movement. The amplitude of movement can only be recorded if the mean distance of the object is constant and calibration is performed by comparison with a known movement amplitude. The device must thus be calibrated separately for each recording.

If the movement of a limited surface is to be recorded, the area can be diminished by using a smaller oscillator coil. An oscillator, working on the transistor principle, can also be constructed. The oscillator can thus be kept down in weight and the device can be set up in the immediate vicinity of the target without weighing on the object itself and would, for instance, permit the recording of radial pulse irrespective of the movements of the limb.

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Reticular Influence upon Thalamic and Cortical Potentials Evoked by Stimulation of the Cat's Tongue.

By

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Abstract.

APPELBERG, B., R. L. KITCHELL and S. LANDGREN. Reticular influence upon thalamic and cortical potentials evoked by stimulation of the cat's tongue. *Acta physiol. scand.* 1959. *45*. 48—71. — The experiments were made on *encéphale isolé* preparations of cats anaesthetized with ether during operation. Potentials evoked by stimulation of the ipsilateral tip of the tongue or the ipsilateral dorsal ascending trigeminal tract were recorded in *nucleus ventralis posteromedialis* (VPM) of the thalamus, and from the surface of the cortical tongue projection area. Effects of arousal stimulation on these evoked potentials are described. Electrical stimulation of the mesencephalic reticular formation did not induce any evoked potentials in VPM or cortex. The potentials evoked from the tongue or from the dorsal ascending trigeminal tract were, however, changed by reticular stimulation and by sensory arousal. The negative component of the thalamic evoked potential was thus gradually reduced in amplitude, disappeared and was replaced by a positive potential. These events were accompanied by a reduction in amplitude and a disappearance of the cortical surface potential.

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The observations are interpreted as evidence of reticular influence upon thalamic relay cells in the specific sensory path from the tongue. Alternative explanations of the observed blockade of this path are discussed.

It is a well established fact that the central nervous system possesses mechanisms by means of which the afferent inflow can be influenced, and that an important role in these mechanisms is played by the reticular formation of the brain stem. Reticular stimulation has been shown to affect the sensory discharge at various levels outside as well as inside the central nervous system. For example, the sensitivity of the receptor itself may be adjusted via the reticular formation, as shown by GRANIT and KAADA (1952) in their study of the gamma fibre control of the muscle spindle. HAGBARTH and KERR (1954) and LINDBLOM and OTTOSSON (1954, 1956) have provided experimental evidence of reticular influence upon synaptic transmission at the spinal level of the somatic afferent pathway. This influence was inhibitory in nature and tonically exerted, as was predicted by LINDBLOM and OTTOSSON (1953). They showed that sectioning of the spinal cord resulted in an increase in amplitude of the cord dorsum potential evoked by stimulation of low threshold cutaneous afferents.

Reticular inhibition of the medullary relays in sensory paths was demonstrated by HERNÁNDEZ-PEÓN and HAGBARTH (1955) and by HERNÁNDEZ-PEÓN and SCHERRER (1955) in experiments on the trigeminal nucleus and by HERNÁNDEZ-PEÓN, SCHERRER and VELASCO (1956) on nucleus gracilis.

As electrical stimulation of certain areas within the reticular formation of the brain stem inhibits synaptic transmission in the somatic afferent pathway even at spinal and medullary levels, it was also to be expected that reticular stimulation would reduce or block thalamic and cortical potentials evoked by peripheral stimulation. Reticular blocking of cortical evoked potentials has been reported by PARMA and ZANCHETTI (1956) and by GAUTHIER, PARMA and ZANCHETTI (1956). A similar effect at the thalamic level was described by MORUZZI and MAGOUN (1949), HERNÁNDEZ-PEÓN, SCHERRER and VELASCO (1956) and by KING, NAQUET and MAGOUN (1957). The last mentioned authors recorded the activity evoked by a peripheral stimulation in the medial lemniscus below the thalamic relay nucleus and in the internal capsule above it. Thus controlling the thalamic input and output they were able to decide whether a reticular influence was exerted also at the

thalamic relay of the somatic afferent pathway. A reduction in amplitude of the evoked potential recorded from the thalamic output was observed during reticular stimulation. A similar effect was, however, obtained in records from the thalamic input. The authors therefore concluded that the reduction in amplitude was due to blocking effects exerted below the thalamus. However, other effects of reticular stimulation were found to be induced at the thalamic level, and it was finally concluded that the thalamic relay nucleus was influenced by the reticular formation of the brain stem. Further evidence of such an influence will be presented in this report. It was observed that arousal stimulation profoundly changed the focal potential evoked in the thalamic relay nucleus by electrical stimulation of the tongue or the dorsal ascending trigeminal tract.

Methods.

Eleven cats were used for the experiments. The animals were anaesthetized with ether during the operation. Local anaesthesia was applied to incisions and pressure points before mounting the animal in the Horsley-Clarke apparatus. The spinal cord was cut or blocked between C 1 and C 2 by injection of a local anaesthetic (xylocain) into the cord. Respiration was maintained artificially. In a couple of control experiments the cord was left intact and the animal immobilized by means of intravenous injection of d-tubocurarine. In some of the experiments small amounts of chloralose (5—10 mg/kg body weight) were injected intravenously in order to produce cortical synchronization, providing a background for the EEG-arousal. In most experiments, however, no attempt was made to synchronize the EEG.

The main features of the experimental arrangements are shown in Fig. 1. Electrical stimulation was applied to the ipsilateral tip of the tongue or to the ipsilateral dorsal ascending trigeminal tract at the mesencephalic level and to the ipsilateral or contralateral mesencephalic reticular formation. Single shocks, 1—3/sec were applied to the trigeminal path, whereas the reticular formation was stimulated at 250/sec using pulses with a duration of 1 msec and a stimulus strength of 5—8 V. Bipolar concentric electrodes were used for stimulation within the brain. They were inserted by means of the stereotactic instrument. Sensory arousal was induced by pinching the pinna, blowing air into the nostrils or squirting Ringer solution into the eye.

Thalamic evoked potentials were recorded via a KCl filled microelectrode with a relatively coarse tip (1—5 μ). The microelectrode was stereotactically guided to the tongue relay area within the *nucleus ventralis posteromedialis* (VPM) as described by APPELBERG and LANDGREN (1958). The nucleus was penetrated by the electrode, which was then withdrawn to a suitable recording position. This was done in order

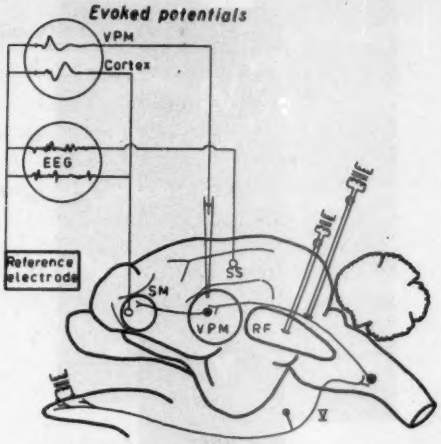

Fig. 1. Diagram showing stimulating and recording arrangements.
VPM = nucleus ventralis posteromedialis of the thalamus.
SM = the tongue projection area of the sensorimotor cortex.
SS = gyrus suprasylvius.
RF = formatio reticularis mesencephali.

to destroy units close to the tip of the microelectrode thereby limiting the distortion of the focal potential induced by nearby cellular activity.

The evoked cortical surface potential was recorded within the tongue projection area. The EEG was also led off from this area and from the middle suprasylvian gyrus. A common reference electrode was screwed in bone over the contralateral sinus frontalis.

Records of the evoked potentials usually consisted of 20—40 superimposed sweeps. Negativity is recorded upwards in all figures.

Recording and stimulation sites were histologically localized using serial sections through the thalamus and the mesencephalon. Further details concerning stimulating and recording procedures as well as surgical and histological techniques are given in previous reports (COHEN *et al.*, 1957, APPELBERG and LANDGREN 1958).

Results.

A. Reticular effects upon potentials evoked by stimulation of the tongue.

The responses evoked in the thalamus and in the cortical tongue projection area by electrical stimulation of the tongue of the cat were changed by electrical stimulation in the mesencephalic retic-

Fig. 2. Records of potentials evoked in nucleus ventralis posteromedialis (VPM) of the thalamus and in the cortical tongue projection area (SM) by electrically stimulating the ipsilateral tip of the cat's tongue. A before, B during, C 20 sec after and D 30 sec after electrical stimulation within the mesencephalic reticular formation (8 V, 1 msec duration, 250/sec during 20 sec). Time 2 msec. Amplifier time constant (TC) 50 msec. The faint spikes seen in records B of this figure as well as in B and G of Fig. 3 and B of Fig. 4 are artefacts due to the reticular stimulation. Negativity is recorded upwards in this and all subsequent figures.

ular formation. The reticular stimulation itself did not evoke any localized thalamic or cortical responses (see Fig. 6 G), unless the stimulus was made strong enough to spread to the medial lemniscus or to the dorsal ascending trigeminal tract.

The change of the evoked cortical surface potential consisted in a considerable reduction of the amplitude of its initial positive phase as well as of the following negative phase. This effect is demonstrated in Fig. 2. The figure shows pairs of simultaneously obtained records from VPM (top beam) and cortical tongue pro-

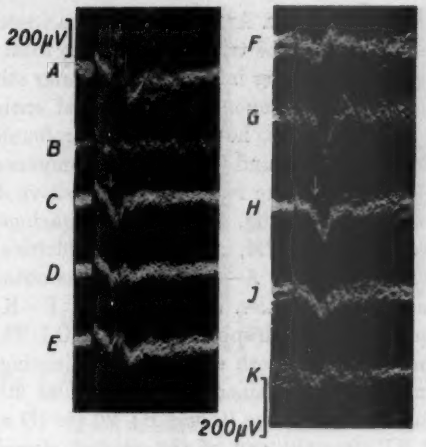

Fig. 3. Records of thalamic focal potentials evoked in VPM by electrical stimulation of the tongue.

A and F, controls before reticular stimulation.
B and G, during reticular stimulation.
C and H, 10 sec after reticular stimulation.
D and I, 20 sec after reticular stimulation.
E and K, 60 sec after reticular stimulation.

Arrows in C and H mark step on falling phase of the positive potential.

A—E obtained from intact curarized preparation.
F—K obtained from *encéphale isolé* preparation.

Time 2 msec. TC: A—E, 50 msec; F—K, 0.5 sec.

jection area (lower beam). The first pair of records (A) was obtained before, and the pair marked B during, electrical stimulation of the mesencephalic reticular formation. C and D were recorded 20 and 30 sec after the end of the reticular stimulation. The amount of reduction in amplitude seen in Fig. 2 B was not typical for all our observations. In some cases the reticular stimulation resulted in a complete disappearance of the cortical potential. In other trials the effect, although always obvious, was less pronounced than that illustrated in Fig. 2. The observations confirm the previous findings of GAUTHIER, PARMA and ZANCHETTI (1956).

The change of the thalamic evoked response induced by reticular stimulation was more complex. Under our experimental conditions the main features of the focal potential evoked in VPM by electrical stimulation of the tongue was a triphasic positive-

negative-positive potential (cf. APPELBERG and LANDGREN 1958). The negative component of the triphasic thalamic focal potential was reduced in amplitude under influence of reticular stimulation, and in several cases this component disappeared entirely (Fig. 2 B and 3 B). In many cases, however, more profound changes occurred. The initial positive and the negative components of the thalamic evoked potential were replaced by a positive deflection. This effect is illustrated in Fig. 3 and 4. All tracings of these figures were recorded from VPM, in response to electrical stimulation of the tongue. The series A—E of Fig. 3 was obtained from an intact, curarized preparation and the series F—K from an *encéphale isolé* preparation with spinal cord cut at C 1. The controls A and F of the two series both showed a dominating negative potential. B and G were obtained during reticular stimulation, and the following records 10 sec (C and H), 20 sec (D and I) and 60 sec (E and K) after the end of the arousal stimulation. In series A—E the thalamic focal potential disappeared during the period of reticular stimulation and a positive deflection not previously seen developed immediately after the end of the reticular stimulation. In the series F—K a similar positive potential developed but it appeared even during the period of arousal. For the sake of convenience this positive deflection will be referred to as "arousal positivity". Its latency was the same as that of the initial positivity of the control records (3 msec in Fig. 3 A and C). Generally the maximum of the arousal positivity was simultaneous in time with the maximum of the negative component of the control. The latency of the two maxima was 5 msec in Fig. 3 A and C. In the series F—K of Fig. 3 the maximum of the arousal positivity appeared slightly earlier than that of the negative component of the control. The latency of the first mentioned maximum was 6 msec and that of the latter 7 msec. The control records of this series did, however, differ from the triphasic type generally observed.

A careful study of Fig. 3 C and H reveals a step on the falling phase of the arousal positivity, which may indicate that this deflection is formed by two components, one corresponding to the initial positivity, and one to the negative component of the triphasic thalamic evoked potential seen in the control records.

A complete reversal of the negative thalamic evoked potential into an arousal positivity was not observed in all our experiments. Out of 75 recorded arousal experiments in 6 animals 31

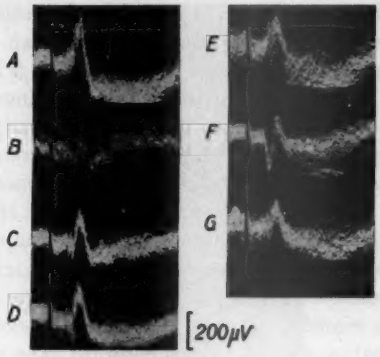

Fig. 4. Records of thalamic focal potentials evoked in VPM by electrical stimulation of the tongue.

A and E, controls before reticular stimulation.
B, during reticular stimulation.
C and F, 10 sec after reticular stimulation.
D and G, 80 sec after reticular stimulation.

Time 2 msec. TC 50 msec.

resulted in a development of an arousal positivity. No effect was seen in 25 experiments. In 19 a reduction of the amplitude of the negative component was observed. The reduction was, however, often accompanied by certain signs of interference between this component and an underlying positive deflection. Such records are shown in Fig. 4 B and F. The control records A and E of the two series did not show any initial positive deflections. During (B) or after (F) reticular stimulation a deep initial positivity developed in parallel with the reduction of the negative component, which appeared to sink down into the trough of a positive deflection.

As described above the changes of the thalamic evoked potential developed gradually during and after the period of arousal stimulation. The effects generally lasted 20 to 60 sec after the end of this period. In two experiments, however, more longlasting effects were observed. Fig. 5 illustrates selected records obtained during one of these experiments. The stimulation was given after the cat had been left undisturbed for about an hour in a silent room. The EEG showed a sleep pattern with synchronized α-activity. Electrical stimulation of the tongue evoked a typical triphasic response in VPM and a positive—negative surface potential in the cortical

tongue projection area (Fig. 5 A). During the reticular stimulation the evoked VPM potential changed into an arousal positivity, of a type similar to that shown in Fig. 5 B, which was recorded 2 min and 40 sec after the end of the stimulation period. The cortical surface potential was simultaneously reduced in amplitude. About 5 min after the end of the arousal period the cortical response to tongue stimulation was almost completely abolished and the thalamic response consisted of a prolonged positive potential (Fig. 5 C). During this stage the microelectrode in VPM was moved up and down through about 1 mm in the relay nucleus. The positive evoked potential of the type shown in Fig. 5 C and D was recorded all over the nucleus where previously a dominating negative deflection was obtained. The changes lasted about 15 min and then the evoked potentials gradually returned to normal. The cortical response seemed to be increased in amplitude towards the end of the experiment (Fig. 5 E).

The electrical stimulation applied to the mesencephalic reticular formation generally lasted 10 to 20 sec. Too short stimulation periods were of no effect and if the reticular stimulation was continued over longer periods the thalamic response returned to normal in spite of the arousal stimulation. When the reticular formation was repeatedly tetanized in the same experimental animal, it was observed that the effects on the evoked potentials decreased. In fact, the most prominent changes were obtained in the first two or three trials of each experiment, or — as was the case in the experiment of Fig. 5 — immediately after a period of rest, when the animal was left undisturbed.

The positive wave of low amplitude and relatively long duration, which follows the negative component of the thalamic focal potential, was also affected by reticular stimulation. Its amplitude generally decreased in parallel with the decrease of the negative component (Fig. 2, 3 and 4). Experiments in which the negative component was completely abolished by the reticular stimulation also showed an extinction of the late positivity (Fig. 2 B and 3 B). These observations suggest a close relationship between the two components.

In the control records a negative hump with a latency of 10 msec is often shooting up from the bottom of the late positivity (cf. Fig. 2 A, VPM). This late negative component of the thalamic focal potential seems to increase in amplitude during reticular stimulation. Thus the control record A of Fig. 4 shows a deep and

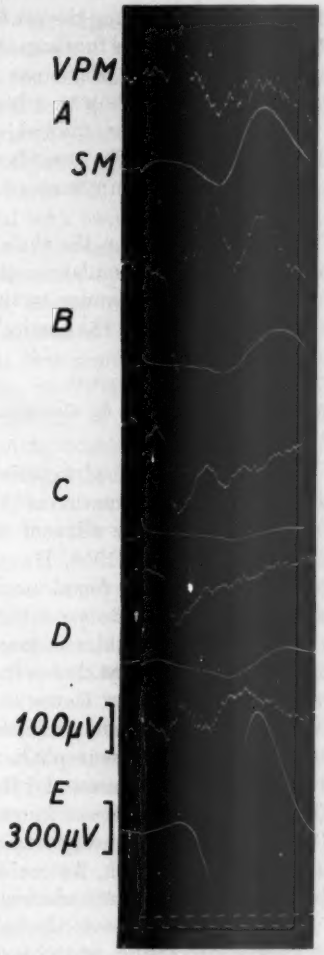

Fig. 5. Records of potentials evoked in VPM and SM by electrical stimulation of the tongue. The changes of polarity and amplitude of the stimulus artefact are due to changed positions of the electrodes stimulating the tongue.

A, before reticular stimulation.
B, 2 min 40 sec after reticular stimulation.
C, 4 min 40 sec after reticular stimulation.
D, 14 min 30 sec after reticular stimulation.
E, 26 min 30 sec after reticular stimulation.

Time 2 msec. TC 0.5 sec.

long lasting late positive component. During the reticular stimulation the time course of this component was fundamentally changed (Fig. 4 B). Its duration was shortened to about one third of that seen in the control, whereas the amplitude was only decreased by one tenth. Moreover its returning phase showed a hump and finally rose slightly above the baseline. The records thus indicate the existence of two interacting late components of the thalamic focal potential.

In one experiment reticular effects upon the thalamic and cortical potentials evoked by electrical stimulation of the contralateral paw were investigated. Changes similar to those observed in the thalamic tongue relay nucleus and the cortical tongue projection area were seen also in this case.

B. Reticular effects upon potentials evoked by stimulation of the dorsal ascending trigeminal tract.

The interpretation of the above described experiments is complicated by the fact that the reticular formation is known to exert an inhibitory influence on the medullary relay of the trigeminal path (HERNÁNDES-PEÓN and HAGBARTH 1955, HERNÁNDEZ-PEÓN and SCHERRER 1955). It was therefore found necessary to investigate the reticular influence upon the potentials evoked by electrical stimulation of the trigeminothalamic tracts. The ipsilateral dorsal ascending trigeminal tract was chosen for stimulation. The course of this tract was described by CARPENTER (1957). It was easily reached by a pair of concentric electrodes parallel to those used for the stimulation of the mesencephalic reticular formation. From the work of BERRY, ANDERSSON and BROOKS (1956), TORVIK (1957) and CARPENTER (1957) it was known that fibres from the dorsomedial part of the main sensory trigeminal nucleus reached the ipsilateral VPM via this path. As could be expected from the results of these investigations, stimulation of the dorsal ascending trigeminal tract evoked a response in the same part of VPM as electrical and tactile stimulation of the ipsilateral tip of the tongue. The cortical surface potential evoked by stimulation of the tract was localized to the tongue projection area and bordering parts of the face projection area. A response of maximum amplitude was recorded within the tongue cortex. It faded away and disappeared in the more distant parts of the facial cortex. Potentials evoked by electrical stimulation of the dorsal ascending trigeminal tract were thus recorded within thalamic and cortical

areas, upon which ipsilateral tactile fibres from the mouth and perioral regions were known to project. It therefore seems reasonable to conclude that this tract mediates tactile fibres from the ipsilateral half of the tongue and mouth cavity as well as from the perioral regions.

The thalamic and cortical responses to tract and tongue stimulation are shown in Fig. 6 A and F. The former consists of a negative potential with two peaks followed by a positive wave and a late negative component of low amplitude. The initial part of the response is distorted by a negative deflection due to the stimulus artefact. The latency was therefore difficult to assess. It was 0.5 msec when measured from the beginning of the artefact to the trough of the first positive deflection. The distance from the level of stimulation to VPM was 6—7 mm, which suggests a central conduction velocity of 12—14 m/sec for the fastest fibres of the dorsal ascending trigeminal tract. The latency of the triphasic potential evoked from the tongue was 3.6 msec, thus showing a difference of 3.1 msec used for peripheral conduction, synaptic delay in the medulla and central conduction up to the mesencephalic level.

The latency measured to the trough between the two negative peaks in Fig. 6 A was 1.8 msec. As shown by the top record in F, the negative component of the triphasic potential evoked from the tongue has two similar peaks. The time interval (1 msec) between these agrees with that measured between the two peaks of the potential evoked from the tract. This indicates that the second peak of the two negative potentials is not due to thalamic stimulation by slower conducting fibres in the afferent path. The initial positive and negative deflections may be interpreted as due to the arrival of the presynaptic volley at the thalamic relay nucleus, whereas the second negative peak may indicate postsynaptic activity. This interpretation does, however, require a rather long synaptic delay of 1.3 msec. The alternative explanation would be that the second peak is due to activity in secondary thalamic neurones.

As previously mentioned, electrical stimulation of the mesencephalic reticular formation did not itself evoke any localized thalamic or cortical responses (Fig. 6 G). The arousal stimulation did, however, change the thalamic potential evoked from the dorsal ascending trigeminal tract. The changes resembled those observed in the thalamic potential evoked from the tongue. They consisted

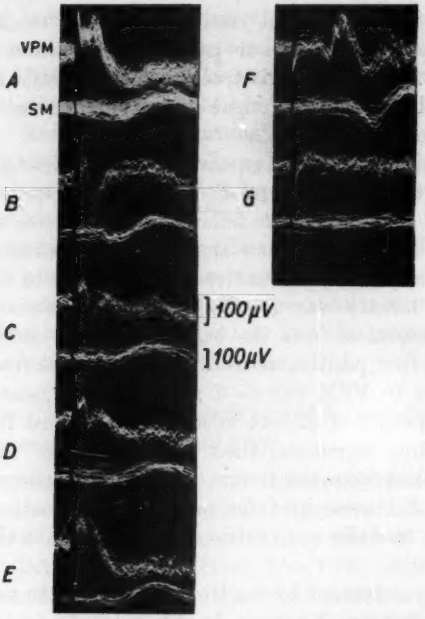

Fig. 6. Records of potentials evoked in VPM and SM by electrical stimulation of the dorsal ascending trigeminal tract (A—E), of the tongue (F) and of the mesencephalic reticular formation (G).

A and F, controls before reticular stimulation.
B, immediately after reticular stimulation.
C, 10 sec after reticular stimulation.
D, 20 sec after reticular stimulation.
E, 13 min after reticular stimulation.

Note: No thalamic or cortical potentials are evoked by single shock stimulation (10 V, 0.1 msec duration, 2/sec) in the reticular formation (G). Time 2 msec. TC 50 msec.

in a reversal of the second negative peak into a positive deflection and of the late positive wave into a negative potential of about 10 msec duration. The effects are shown in Fig. 6 B which was obtained immediately after the end of the reticular stimulation. The latency and the duration of the new positive deflection agreed with those of the second negative peak of the control record. The first peak of the thalamic potential evoked from the tract was unaffected by reticular stimulation.

The thalamic evoked potential gradually returned to normal

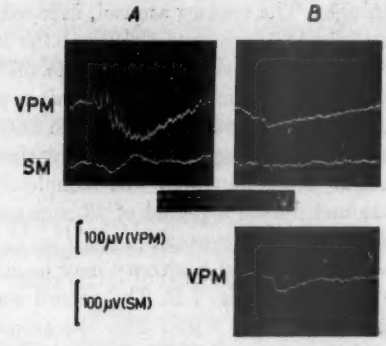

Fig. 7. Records of potentials evoked in VPM and SM by electrical stimulation of the tongue. A before and B after sensory arousal induced by squirting Ringer solution into the cat's eye. Time 5 msec for upper records and 2 msec for lowest record in column B. TC 0.5 sec.

during a period of about 10 min. Intermediary stages, recorded 20 and 30 sec after the end of the reticular stimulation, are shown in records C and D of Fig. 6. A slight reduction of the cortical surface potential accompanied the changes in the thalamus.

C. The effect of sensory arousal upon the thalamic evoked potential.

The changes of the thalamic evoked potential described above were also found when the electrical stimulation of the mesencephalic reticular formation was replaced by peripheral stimulation, for instance pinching of the pinna, blowing air into the nostrils or squirting Ringer solution into the eye. Effects were observed on the thalamic potential evoked from the tongue as well as from the dorsal ascending trigeminal tract.

In the experiment from which the records of Fig. 7 were obtained, a sequence of events similar to that illustrated in Fig. 5 was induced by reticular stimulation. When control conditions were again established (Fig. 7 A), Ringer solution was squirted into the cat's eye. The result is shown by the records in Fig. 7 column B. The thalamic evoked potential was, in this case, recorded before destruction of nearby unitary activity, and electrical stimulation of the tongue discharged a thalamic cell on top of the negative component of the focal potential. This cell also responded to

touch of the cat's tongue. The sensory arousal, induced by squirting Ringer solution into the eye, thus abolished the unitary activity as well as the negative component of the thalamic focal potential. The only response obtained from VPM was now a positive potential, which increased somewhat in amplitude during the following five minutes. Simultaneously the evoked surface response in the cortical tongue projection area was completely blocked. These changes remained during a period of 12 min and then the potentials gradually returned to normal.

The time course of the arousal positivity may be studied more closely in the lower record of Fig. 7 B. This record was obtained with a faster sweep speed about 5 min after the arousal stimulation. The latency of the potential was 3.4 msec and it rose to a maximum in 1 msec. Its duration was 9 msec. The latency agrees with that of the initial positive phase of the VPM record of Fig. 7 A, which was also 3.4 msec. The thalamic focal potential shown in Fig. 7 A is distorted by nearby unitary activity. When recording the arousal positivity the microelectrode was, however, moved up and down through the nucleus and the positive potential was found all over the area where previously a triphasic potential was observed.

D. EEG effects.

Electrocorticograms were obtained from the tongue projection area of the sensorimotor cortex and from the middle part of the suprasylvian gyrus. In response to electrical stimulation of the mesencephalic reticular formation these electrocorticograms showed the well known signs of cortical arousal, as described by MORUZZI and MAGOUN (1949). Thus the slow α-rhythm, when present in the control, disappeared during reticular stimulation and did not return until a few seconds after the stimulation had been discontinued (Fig. 8 A—C). In most of our experiments, however, no α-rhythm was seen in the EEG, which showed general "desynchronization". On the contrary a regular rhythm of potential variations developed in response to reticular stimulation (Fig. 8 E). This synchronous activity, which was observed in the majority of our experiments, was recorded within the sensorimotor cortex. The amplitude of the waves was about 200 μ V and the frequency 20—30/sec. They were more sensitive to anaesthesia than were the evoked potentials of the tongue projection area, and disappeared after injection of small amounts (10 mg/kg) of chloralose

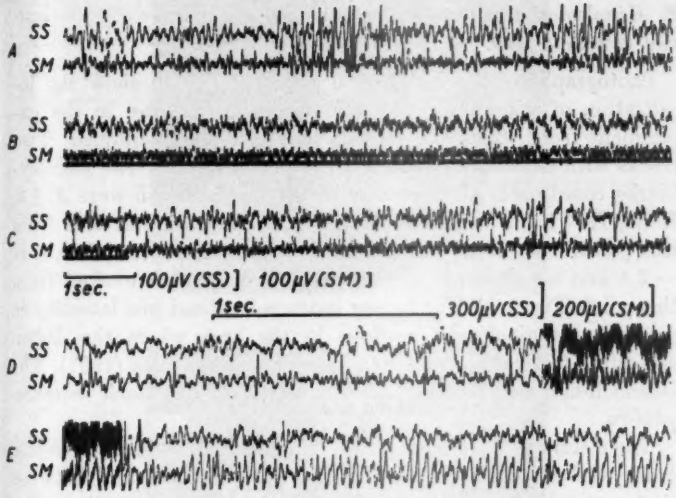

Fig. 8. Electrocorticograms recorded from gyrus suprasylvius (SS) and from the tongue projection area within the sensorimotor cortex (SM). Reference electrode screwed in bone over the contralateral sinus frontalis. Reticular stimulation indicated by signal beam below SM in B and C and by artefacts on SS in D and E. A—E show disappearance of α-rhythm during and after reticular stimulation. D and E show development of rhythmic activity (30/sec) in SM during and after reticular stimulation.

intravenously. From the suprasylvian gyrus a less pronounced and less regular rhythmical activity was sometimes recorded in response to reticular stimulation, but generally this lead only showed a "desynchronization".

The rhythmical activity induced in the sensorimotor cortex resembles the recruiting response evoked by stimulation of the intralaminar nuclei (cf. MORISON and DEMPSEY 1942). According to BRODAL (1957) long axons from reticular neurones ascend in the central tegmental tract to reach the intralaminar nuclei and centrum medianum. As our stimulating electrodes in the mesencephalic reticular formation were close to the area occupied by the central tegmental tract, it is possible that the stimulus may have reached the ascending fibres to the intralaminar nuclei thereby inducing a recruiting response.

E. Histological localization of stimulating and recording sites.

Photographs of the histological sections (Fig. 9) show the localization of stimulating (A) and recording (B) sites in the experiment from which the records of Fig. 6 were obtained. Two tracks with electrocoagulations are seen in Fig. 9 A. The Horsley-Clarke coordinates of the centre of the medial lesion were A 2.5, L 2.5, H— 1.5. The potentials of Fig. 6 A—E were evoked from this point. The lateral lesion has the coordinates A 2.5, L 3.5, H — 2.5 and no thalamic or cortical potentials were evoked from this point (Fig. 6 G). The former lesion is localized just laterally to *fasciculus longitudinalis medialis* in the area where the dorsal ascending trigeminal tract was found by CARPENTER (1957). The lateral lesion was localized within *substantia reticularis mesencephalica.*

The electrode sites, from which the dorsal ascending trigeminal tract was stimulated, were in other experiments found between A 2 and 3, L 2 and 2.5 and H — 1 and — 2. The coordinates of the stimulating points within the mesencephalic reticular formation varied from A 1 to A 3, L 2.5 to L 3.5 and H — 1 to H — 3.

If the stimulating electrodes were inserted 4.5 mm laterally to the midline evoked potentials, similar to those of Fig. 6 A, were again obtained at a horizontal level comparable to that of the lowest point in the lateral track of Fig. 9 A. The response was interpreted as due to stimulation of the medial lemniscus. These observations indicate that the volume reached by our stimulus was below 2 mm in diameter as no evoked potentials were seen when the electrodes passed between 3 and 4 mm laterally to the midline.

The arrow in Fig. 9 B indicates the faint track left by a microelectrode entering VPM. The thalamic records of Fig. 6 were obtained from this track. The coordinates of the tip of the microelectrode were A 9, L 4, H 0. It was situated within the medial half of VPM and in the caudal third of the ventrobasal nuclear complex. A similar localization of the recording sites were found in all our experiments.

Discussion.

The above described experiments have added new evidence of a reticular influence exerted at the thalamic level of the specific afferent pathway from the tongue. The two main observations

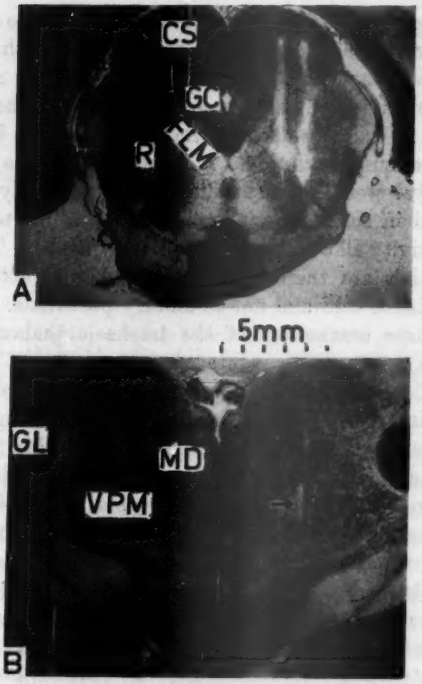

Fig. 9. Frontal sections through mesencephalon (A) and thalamus (B) of the cat used in the experiment of Fig. 6. Note stimulating electrode tracks in A, and recording microelectrode track marked by the arrow in B.

CS: colliculus superior;
FLM: fasciculus longitudinalis medialis;
GC: griseum centrale;
GL: corpus geniculatum laterale;
MD: nucleus medialis dorsalis;
R: substantia reticularis mesencephalica;
VPM: nucleus ventralis posteromedialis.
100 μ, Toluidine blue.

requiring interpretation are the reticular blockade of the thalamic and cortical evoked potentials and the reversal of the negative component of the triphasic thalamic evoked potential.

The possibility that the reversal was due to a change from extracellular to intracellular recording of the action potential of cells

lying close to the tip of the microelectrode, may be excluded as particular precautions were taken to eliminate such distortions. Thus a rather coarse microelectrode was used, and records were taken on the way up in the microelectrode track. The spinal cord was blocked in order to diminish movements of the brain caused by blood pressure variations. No sudden changes of the DC potential level were observed during the gradually developing reversal, and in some experiments it was possible to record the arousal positivity all over the thalamic relay nucleus. The recorded focal potentials are therefore no doubt due to activity within pools of thalamic cells and not to unitary potentials.

The negative component of the triphasic thalamic potential evoked from the tongue was interpreted as a sign of postsynaptic activity in the thalamic relay nucleus (APPELBERG and LANDGREN 1958). The interpretation was based on the following arguments: 1. The response showed a focal localization within the VPM; 2. Thalamic units responding to physiological stimulation of the tongue were generally found in the area of VPM, where the negative component of the focal potential was maximal in amplitude. In response to electrical stimulation these units were usually discharged on the crest of the negative component.

This sign of postsynaptic activity in the relay nucleus is now seen to diminish and gradually reverse under the influence of reticular activation. Simultaneously the evoked cortical surface potential is reduced in amplitude or disappears. The gradual replacement of the negative postsynaptic wave by a potential of opposite polarity calls to mind the interaction between excitatory and inhibitory postsynaptic potentials in the motoneurone, which was so beautifully demonstrated by BROCK, COOMBS and ECCLES (1952). If the negative component of the triphasic thalamic focal potential is interpreted as due to excitatory and the arousal positivity to inhibitory postsynaptic activity in the same pool of thalamic cells, the hypothetical mechanism illustrated in Fig. 10 may be presented in order to explain our observations.

The stimulus whether applied to the tongue or to the dorsal ascending trigeminal tract is thought to activate two paths, one excitatory and one inhibitory, which end on the thalamic cells assumed to be responsible for the recorded potential reversal (Fig. 10 A). The two paths could be due to a branching of the excitatory inflow to the thalamus as shown in Fig. 10. The branching could take place at lower levels provided the two paths travelled together

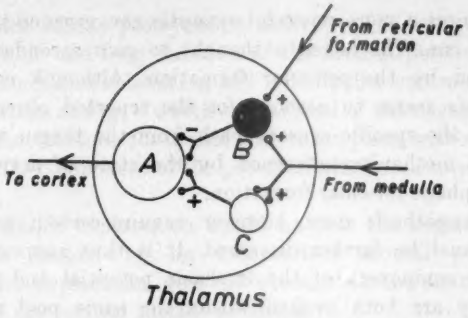

Fig. 10. Diagram showing postulated mechanism responsible for the reticular blockade of the specific sensory path from the tongue. + indicates excitatory and — inhibitory synapses.

in the dorsal ascending trigeminal tract. An interneurone (Fig. 10 B) is required in the inhibitory path in order to change excitatory synaptic transmission into inhibitory (cf. ECCLES, FATT and LANDGREN 1956), and in order to provide a locus for interaction between the specific afferent inflow and fibres from the reticular formation. These fibres are assumed to facilitate the inhibitory interneurone. They cannot exert their inhibitory action directly upon the cells represented by A in Fig. 10 because no thalamic evoked potential is seen in response to reticular stimulation. The inhibitory interneurone need not necessarily be located in VPM but it should lie above the level where the dorsal ascending trigeminal tract was stimulated.

An interneurone is also drawn in the excitatory path. This was done in order to account for the similar latencies of the postulated inhibitory and excitatory potentials. As previously described in section B a rather long delay (1.3 msec) was observed between the arrival of the fastest afferent impulses in VPM and the occurrence of the potential component interpreted as due to postsynaptic activity. The delay seems to be long enough for another thalamic synapse in the excitatory path. This is not in agreement with the conclusion of APPELBERG and LANDGREN (1958) that there was only one thalamic synapse in the specific path from the tongue. A more detailed analysis of the time course of the thalamic evoked potential may, however, contribute further evidence.

Under certain conditions the excitatory path should provide a channel of low resistance through the thalamus. This path there-

fore requires a more powerful synaptic convergence than the inhibitory one. The latter is thought to gain ascendancy only if facilitated by the reticular formation. Although complex the hypothesis seems to account for the reported observations. It provides the specific sensory path from the tongue with a self-blocking mechanism governed by the state of activity in the mesencephalic reticular formation.

The hypothesis does, however, require certain assumptions, which must be further discussed. It is thus assumed that the negative component of the triphasic potential and the arousal positivity are both evoked within the same pool of thalamic neurones, and that a direct causal relationship exists between the arousal positivity and the blockade of the evoked potentials. These assumptions are not firmly established. A causal relationship between the two last mentioned events rests on their simultaneous occurrence. This is, of course, no proof, and the possibility that the two phenomena are completely unrelated cannot be excluded.

The assumption that the negative component of the triphasic potential and the arousal positivity are evoked within the same pool of thalamic cells is in agreement with the fact that they are evoked by the same peripheral stimulus, and that they appear with similar latencies and time courses. Nevertheless our assumptions require further support from extracellularly or preferably intracellularly obtained records of the activity of thalamic cells.

If one rejects the assumption of a causal relationship between the arousal positivity and the blockade of the afferent path, an explanation of the findings must be sought along other lines. The positivity recorded in VPM may be due to a depolarization of neurones with processes ending in VPM but with cell bodies in a nearby nucleus. The depolarization of this group of neurones is assumed to be caused by the volley in the specific afferent path from the tongue facilitated by impulses from the mesencephalic reticular formation. The positivity in VPM may be induced by current flow from the terminals of the cell processes to the regions of the cell bodies. The blockade of the cortical evoked potential would have to be explained as reticular inhibition exerted at thalamic or cortical levels.

As the potentials here studied were recorded from pools of cells giving rise to interfering action potentials, the arousal positivity could be simply explained as the presynaptic potential revealed

after blockade of the postsynaptic activity. The comparatively large amplitude and long duration of the arousal positivity does not favour such an explanation and it is further contradicted by the observations of Fig. 6 where the reversal of the negative component of the thalamic focal potential takes place without interfering with the component of the potential attributed to presynaptic activity.

Another simple explanation would be that the negative component was masked by an independent arousal positivity. However, this explanation does not account for the simultaneous reduction of the cortical surface potential.

It may also be discussed whether the reduction of the thalamic evoked potential is due to inhibition of the relay cells or to maximal reticular activation of these cells making them unresponsive to further stimulation. The latter interpretation is, however, complicated by the observation of the arousal positivity, unless this is assumed to take place in a quite independent group of neurones, not maximally discharged by the reticular stimulation. Further studies of the activity in single thalamic units may contribute to the solution of this problem.

The idea of the reticular formation as an unselective activator of the central nervous system has more and more been abandoned in favour of theories attributing higher degrees of specialisation to the reticular organization. The observations of reticular inhibition exerted at different levels of the somatic afferent pathway are in accordance with the notion of a differentiated reticular function. It is also likely that this inhibition is selectively exerted upon the different pathways from the periphery to the cortex. Such a mechanism could select the information to be presented to the cortex. Pathways transmitting information of less importance could be blocked thereby focusing the attention upon other more important sources of afferent inflow.

Summary.

1. Effects of arousal stimulation on thalamic and cortical evoked potentials are described. The experiments were made on *encéphale isolé* preparations of cats anaesthetized with ether during operation. The mesencephalic reticular formation was stimulated electrically (5—8 V, 250/sec, 1 msec pulse duration). Potentials evoked

by stimulation of the ipsilateral tip of the tongue or the ipsilateral dorsal ascending trigeminal tract were recorded in *nucleus ventralis posteromedialis* (VPM) of the thalamus, and from the surface of the cortical tongue projection area.

2. Stimulation of the mesencephalic reticular formation did not induce any evoked potentials in VPM or cortex. The potentials evoked from the tongue or from the dorsal ascending trigeminal tract were, however, changed by reticular stimulation and by sensory arousal. The negative component of the thalamic evoked potential was thus gradually reduced in amplitude, disappeared and was replaced by a positive potential. These events were accompanied by a reduction in amplitude and a disappearance of the cortical surface potential.

3. The observations are interpreted as evidence of reticular influence upon thalamic relay cells in the specific sensory path from the tongue. Alternative explanations of the observed blockade of this path are discussed. A hypothesis is presented according to which the blockade is due to reticular facilitation of an inhibitory link in the specific path to the thalamus.

4. It was shown that electrical stimulation of the dorsal ascending trigeminal tract evoked ipsilateral responses in the areas of VPM and cortex upon which tactile fibres from the tongue were previously known to project. It is concluded that tactile fibres from the mouth and the perioral region are mediated through the ipsilateral dorsal ascending trigeminal tract.

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Motor Unit Territory in Different Human Muscles.

By

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Abstract.

BUCHTHAL, F., F. ERMINIO and P. ROSENFALCK. Motor unit territory in different human muscles. *Acta physiol. scand.* 1959. *45*. 72—87. — The spread of action potentials from motor units of different human muscles was studied by means of a twelve lead multielectrode. The occurrence of a positive-negative deflection in the potential of less than 0.2 msec duration indicated the presence of active muscle fibres in the immediate vicinity of the recording lead. In this way the fibres of a motor unit were found to be confined to a circular area of 5—7 mm diameter for the muscles of the upper extremities and of 7—10 mm in the lower extremities. Such areas allow space for the fibres of 25 motor units each comprising 500—2,000 fibres. Within the same muscle or the same type of muscle the diameter of the motor unit territory varied by a factor of four. The maximum voltage within the motor unit increased linearly with the territory. The different average values of maximum voltage and territory in different muscles are interpreted in terms of differences in fibre concentration and total number of fibres per motor unit.

[1] Working with a fellowship from the Rask-Örsted Foundation, Copenhagen.

The territory of a motor unit is not amenable to investigation by histological techniques. Therefore, the disposition of the fibres of a motor unit was determined by mapping the cross-sectional distribution of its action potentials (BUCHTHAL, GULD and ROSENFALCK 1957 b). In the human brachial biceps the fibres of a motor unit were found confined to a relatively small area of the total cross section of the muscle. It was the aim of the present study to determine to what extent these findings apply to other muscles.

In addition the concentration of muscle fibres within the motor units in different muscles is inferred from the maximum voltage within the motor units.

Method.

The multielectrode.

The same type of multielectrode was employed as previously described (BUCHTHAL, GULD and ROSENFALCK 1957 b). There were twelve leads, each 1.5 mm long and 0.5 mm apart in a stainless-steel needle 1 mm in diameter. The electrode, inserted perpendicularly to the fibre direction, recorded from all fibres of a motor unit along its track. The depth of insertion was adjusted to record the potential of maximum voltage on one of the centre leads.

Whenever possible insertions were made both anterior-posteriorly and medio-laterally. With random insertions there is a possibility that the electrodes traverse the periphery of the motor unit. If the fibres are distributed over an approximately circular area (BUCHTHAL, GULD and ROSENFALCK 1957 b), with insertions in the periphery of the motor unit the territory found would be smaller than the actual one. This possible error is evaluated below (p. 83).

The indifferent electrode.

The action potentials were recorded between the single leads of the multielectrode and an indifferent electrode. It was essential that the indifferent electrode be situated in a region from which no potentials are recordable from the motor unit under investigation. Therefore, one of the extreme outside leads of the multielectrode was used as indifferent electrode.

An example of a multielectrode recording of action potentials from the same motor unit is shown in Fig. 1 a. The lead at the largest distance from the tip (lead 12) was used as indifferent electrode. Potentials were confined to leads 3 to 7 while leads 1, 2 and 8 to 11 did not pick up potentials from this motor unit. The absence of potentials at these leads indicates that the indifferent

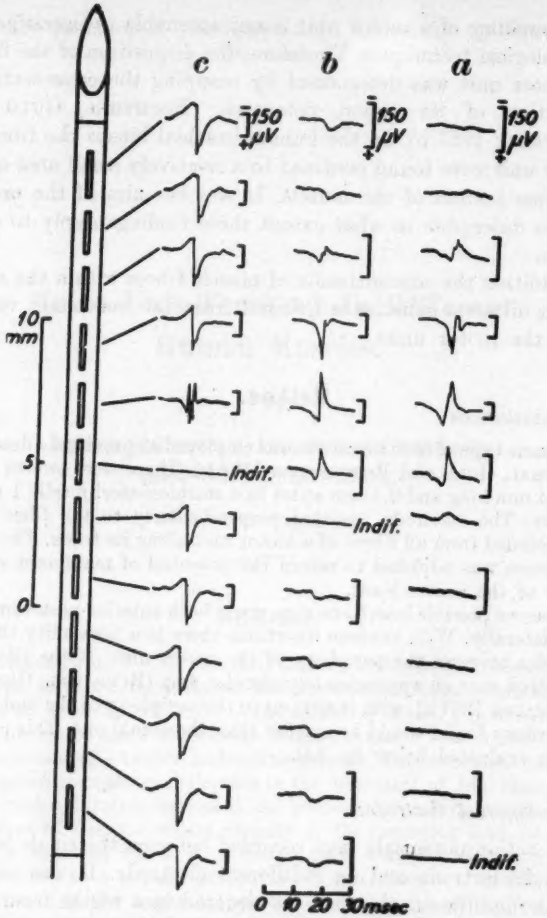

Fig. 1. Influence of a potential picked up by the indifferent electrode on the action potentials recorded by the different leads of the multi-electrode.

a: Indifferent electrode situated at some distance from the motor unit. Responses appear on five leads only.

b: Indifferent electrode situated in the periphery of the same motor unit (lead 7). Identical responses appear on leads 1 and 2 and 8—12. These potentials are picked up by the indifferent electrode.

c: Indifferent electrode (lead 6 from the tip) situated in the centre of the same motor unit. The response appears identical on all leads except lead 5. The reversed direction of the steep deflection of the potential indicates that it is picked up by the indifferent electrode.

lead itself did not record potentials from the motor unit under investigation.

The artifact introduced when the indifferent electrode records potentials is illustrated in Fig. 1 b and c. If the indifferent electrode (lead 7, Fig. 1 b) picks up a potential of low voltage the distortion caused by it cannot be discriminated in the recordings from the central leads but appears on the outer leads. It is evident from Fig. 1 a that the potentials on lead 1, 2 and 8 to 12 in Fig. 1 b arise from the potential recorded by lead 7. When picked up by the indifferent electrode the potentials have a practically constant voltage on the outer leads and their sign is often the reverse of the usual in that the steep deflection of the potentials goes from negative to positive. If the indifferent electrode is situated in a region of the motor unit where high voltage potentials are recorded (*e. g.* lead 6), the potential picked up by it simulates the presence of potentials of equal voltage on all leads except those adjacent to the indifferent electrode and of reversed sign (Fig. 1 c). The complex shape of the potential when recording from lead 5 represents the difference between the potentials recorded by leads 5 and 6 which contain high voltage spikes slightly displaced in time.

The procedure.

The multielectrode was inserted and by slight displacements in depth and small rotations adjusted so that the potential was recorded at highest voltage on lead 5 or 6 from the tip.

For selection of a lead suitable as indifferent electrode the potential was recorded between leads 1 and 12 with lead 12 as indifferent electrode. If no potential was recorded either one was suitable as indifferent electrode. If a spike potential was recorded, it was necessary to displace the multielectrode either deeper into the muscle or to withdraw it according to whether the spike was recorded at lead 1 (steep deflection positive — negative) or at lead 12 (steep deflection negative — positive). If a low voltage potential without spike was recorded, the potential was observed as recorded between leads 1 and 2 and between 11 and 12. According to which of these pairs recorded the lowest potential, lead 1 or 12 was selected as indifferent electrode.

The lead which recorded the potential of highest voltage was kept in constant connection with one channel of the electromyograph (three-channel DISA electromyograph) allowing identification of the potentials belonging to the same motor unit. The other leads were then successively switched to the other two channels. The switch used for this purpose has been described (Buchthal, Guld and Rosenfalck 1957 a). To obtain a true average of motor unit territory it was necessary to scan the muscle by as many insertions as possible. A complication in the identi-

fication of the potentials belonging to a given motor unit is the striking similarity in shape which often characterizes potentials from different motor units recorded on the same lead of the multielectrode. It has been shown that these overlapping units frequently have the same territory (BUCHTHAL, GULD and ROSENFALCK 1957 b).

The potentials were recorded on single sweeps with a time base of 1 mm per msec., a sweep frequency of 5 per sec. and a paper speed of 5 cm per sec. The potentials on each lead were recorded about five times, to allow elimination of random disturbances from potentials of other motor units. The voltage of the motor unit potentials was measured peak to peak and the total duration as the time interval between the initial deflection from the base line and the point at which the terminal deflection again returns to the base line.

The material.

The eight different muscles studied were:

m. biceps brachii
m. deltoideus
m. extensor digitorum communis
m. opponens pollicis
m. rectus femoris
m. biceps femoris
m. tibialis anterior
m. extensor digitorum brevis.

In all, 79 muscles were investigated in 62 subjects without signs or symptoms of neuromuscular disorder. With one exception, a thirteen year old boy, they were between 18 and 65 years of age. The subjects were instructed to sustain a constant voluntary effort during the recording from all leads of the multielectrode.

With a given position of the multielectrode up to six different motor units could be discriminated, for which the maximum voltage was recorded on the central leads. However, since not all of them remained active during the scanning procedure, from each muscle an average of 6 different motor units were investigated in three to five different insertions. Insertions were made within and outside the zone of innervation. Data as to the site of innervation had been histologically determined in different muscles[1]. To ascertain a placement of the electrode transversely to the fibre direction, fibre length, fibre direction and the entry of the nerve branches into the different portions of the muscle were studied in a number of autopsies.

Results.

Motor unit discharges in different muscles as recorded by the leads of the multielectrode are shown in Fig. 2. Potentials con-

[1] Unpublished observations kindly provided by Dr. E. Christensen.

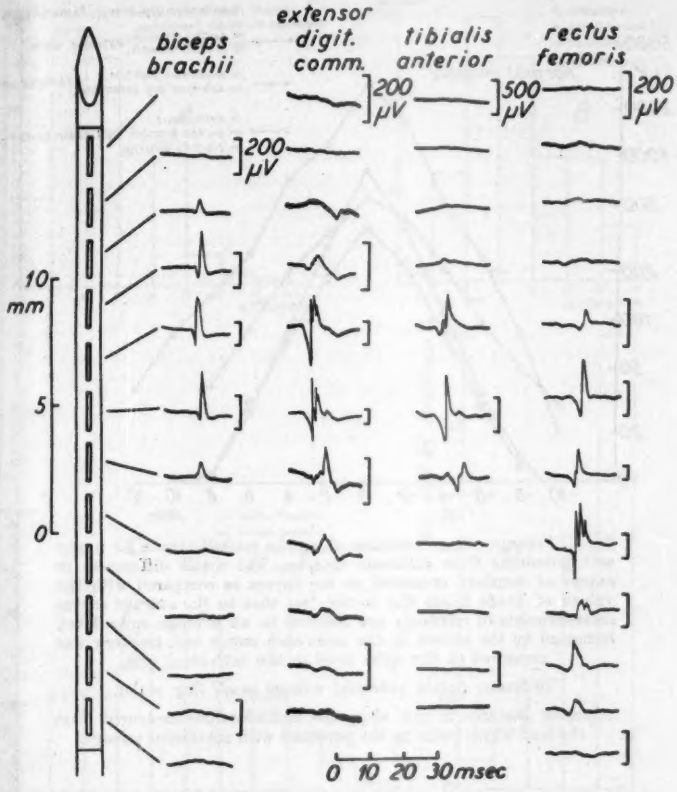

Fig. 2. Motor unit potentials from different muscles recorded by the leads of a multielectrode.

taining a steep deflection with a duration of less than 0.2 msec (denoted as spike) were recorded on three to six leads corresponding to four to ten mm of the cross section of the muscle. The average voltage variation over the cross section of a motor unit is given for different muscles in Fig. 3. They are arranged in four groups according to territory and maximum voltage. In about ten per cent of the motor units examined the voltage variation along the multielectrode had not a single but two maxima; in the m. deltoideus and the m. extensor digitorum communis,

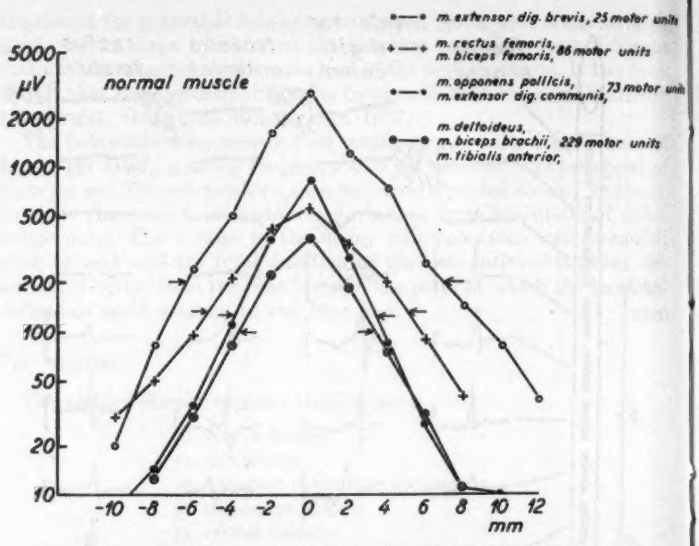

Fig. 3. Average voltage variation along the multielectrode for motor unit potentials from different muscles. The small differences in extent of territory measured on the curves as compared with the values of Table 1 are due to the fact that in the average curves measurements of territorry are referred to an average spike level, indicated by the arrows. In the table each motor unit territory was measured at the spike level of the individual unit.

Ordinate: Action potential voltage in μV (log. scale).

Abscissa: Distance in mm along the multielectrode measured from the lead which picks up the potential with maximum voltage.

however, two voltage maxima were present in only two and four per cent of the motor units.

The area over which the action potentials of a motor unit contained spike components was used as a gauge of the territory over which the fibres of the motor unit was distributed since a spike signals the presence of active muscle fibres in the immediate vicinity of the lead (BUCHTHAL, GULD and ROSENFALCK 1957 a). Thus, in the brachial biceps the muscle fibres giving rise to a spike potential of 50μV are at a distance of less than one mm from the recording lead. In the majority of muscles investigated the voltage level at which spikes appeared in the action potentials was 50 to 100μV. In the m. opponens pollicis and m. biceps femoris the spike level was 150μV and in the m. extensor digitorum brevis it

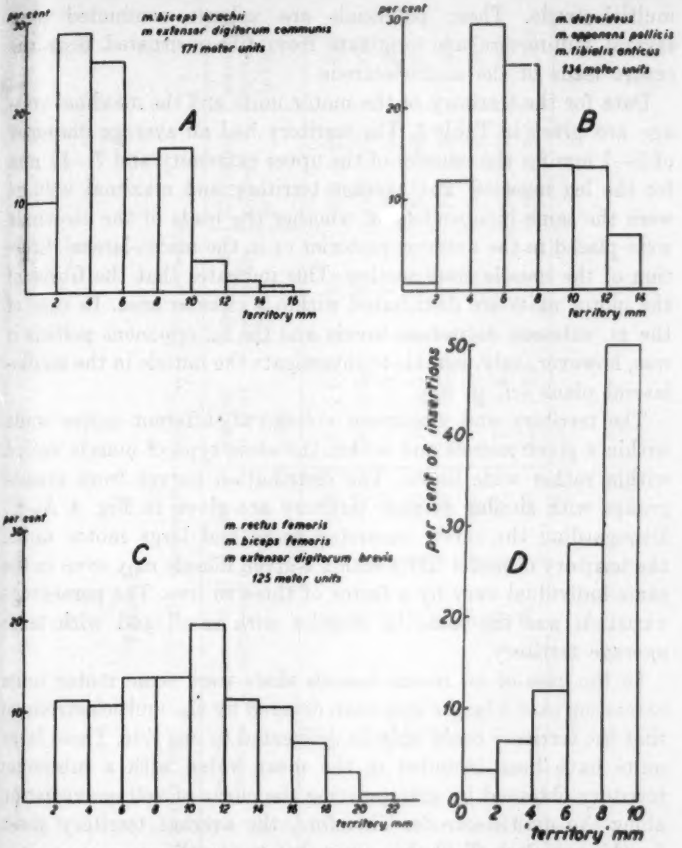

Fig. 4. A—C: Distribution of motor unit territories in different muscles. D: To illustrate the possible influence of eccentric insertions of the multielectrode in the motor units. Distribution of motor unit territories calculated from random insertions of the multielectrode in motor units of identical territory.

Ordinate: A—C: percentage of motor units for each range of territory.
Ordinate: D: percentage insertions for each range of territory.

Abscissa: territory in mm.

was 200μV. The different spike levels are indicated by the arrows on the curves of Fig. 3. Due to spread of the action currents to the medium surrounding the active muscle fibres, low voltage potentials without spikes are recorded by the peripheral leads of the

multielectrode. These potentials are volume conducted over several millimeters and originate from fibres situated near the centre leads of the multielectrode.

Data for the territory of the motor units and the maximal voltage are given in Table I. The territory had an average diameter of 5—7 mm for the muscles of the upper extremity and 7—11 mm for the leg muscles. The average territory and maximal voltage were the same independent of whether the leads of the electrode were placed in the anterior-posterior or in the medio-lateral direction of the muscle cross section. This indicates that the fibres of the motor units are distributed within a circular area. In case of the m. extensor digitorum brevis and the m. opponens pollicis it was, however, only possible to investigate the muscle in the medio-lateral plane (cf. p. 84).

The territory and maximum voltage of different motor units within a given muscle and within the same type of muscle varied within rather wide limits. The distribution curves from muscle groups with similar average territory are given in Fig. 4 A—C. Disregarding the rarely occurring small and large motor units, the territory of motor units within a given muscle may even in the same individual vary by a factor of three to five. The percentage variation was the same in muscles with small and with large average territory.

In the case of m. rectus femoris there were some motor units extending over a larger area than covered by the multielectrode so that the territory could only be delineated to one side. These large units have been included in the mean value with a minimum territory obtained by extrapolating the curve of voltage variation along the multielectrode. Therefore, the average territory given for this muscle in Table I is somewhat too small.

Within the same type of muscle there was a correlation between the territory of the motor unit and its maximum voltage in that maximum voltage increased linearly with increasing territory (Fig. 5).

Territory and maximum voltage did not show significant variations with age of the subject in the age groups investigated (13—65 years). Nor did they vary with the degree of development of the individual muscle. Territory and maximum voltage of a motor unit did not vary systematically according to whether it was the one which appeared firstly on innervation or whether it appeared with stronger effort. This has been ascertained by comparing for

Table I.

Mean values of motor unit territory and maximum voltage in normal muscles.

Muscle	number of muscles	number of motor units	spike level (μV)	territory at spike level (mm)	standard deviation (mm)	maximum voltage (μV)	standard deviation (μV)
Biceps brachii	24	129	100	5.1 ± 0.2	2.4	370 ± 17	190
Deltoideus	7	52	100	6.7 ± 0.4	3.0	450 ± 27	190
Extensor digitorum communis	11	43	100	5.5 ± 0.3	2.1	800 ± 59	390
Opponens pollicis	10	34	150	7.4 ± 0.4	2.6	1,000 ± 83	500
Rectus femoris	9	65	100	(10.0 ± 0.6)	4.6	550 ± 38	300
Biceps femoris	5	35	150	8.8 ± 0.7	4.1	900 ± 67	400
Tibialis anterior	8	47	100	7.0 ± 0.4	3.0	620 ± 43	300
Extensor digitorum brevis	5	25	200	11.3 ± 0.8	4.1	3,000 ± 300	1,500

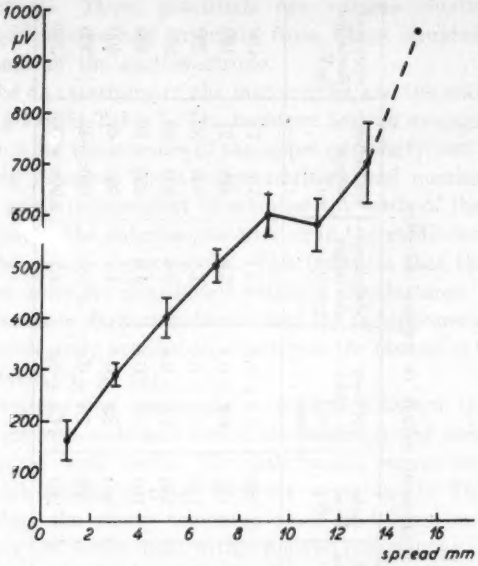

Fig. 5. Correlation between maximum voltage and territory for 324 motor units (m. deltoideus, m. biceps brachii, m. extensordigitorum communis, m. tibialis anterior and m. rectus femoris). Vertical lines indicate mean error.

Ordinate: maximum voltage in μV.
Abscissa: territory in mm.

each muscle average values obtained from ten to twenty different motor units at weak effort with values from a similar number of motor units at stronger effort. The territory and maximum voltage of motor units giving rise to polyphasic potentials did not deviate from those with less complex shapes of potential.

The question arises whether the large variation in territory and maximum voltage in the individual muscle is a true expression of differences between the motor units or whether it is due to what portion of the motor units the multielectrode traverses. Even if insertions in the very periphery of a motor unit simulate units with small territory, the probability for such insertions is so small that eccentric insertions of the multielectrode only are a subordinate determinant of the variation in motor unit territories. This is evidenced by the distribution calculated on the assumption

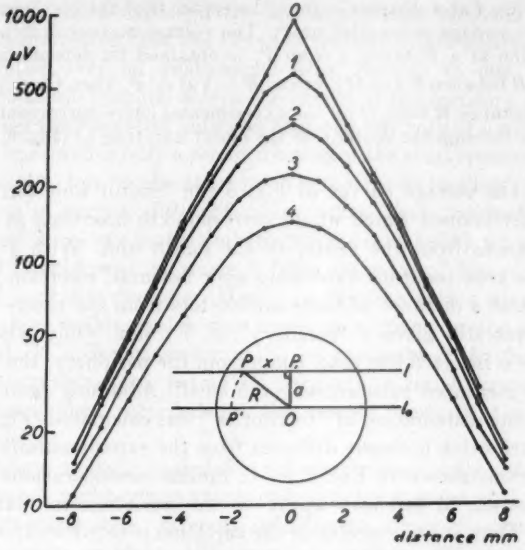

Fig. 6. Voltage variation along the multielectrode calculated for insertions at different distances from the centre of the same motor unit as indicated (in mm) by the figures on the curves (curves 1—4). The calculation is based on the experimental curve (0) derived from 81 motor units with high maximum voltage assumed to represent insertions of the electrode through the centre part of the units (m. deltoideus, m. biceps brachii, m. extensor digitorum communis, m. tibialis anterior and m. rectus femoris).

Inset: To illustrate the determination of the voltage variation along a line l at a distance a from the centre O of a motor unit from the variation along a line l_0 through the centre.

of random insertion of the multielectrode in identical motor units of circular territory. To this purpose the territory was calculated for insertions of the multielectrode at different distances from the centre of a motor unit. As a basis for this calculation action potential recordings with high values of maximum voltage were used in that it seemed justified to assume that these recordings correspond to insertions through the centre part of the motor unit. The voltage variation along lines outside the centre of the motor unit is illustrated in Fig. 6. It was calculated in the following way:

For a line l at a distance a from the center O of the motor unit the maximum voltage is recorded at P_1. The voltage measured at point P on this line at a distance x from P_1 is obtained by determining the distance R between P and O given as $R = \sqrt{x^2 + a^2}$. Then the voltage is read at distance R from O on the experimental curve corresponding to insertions through the center O of the motor unit (line l_0) (Fig. 6, inset).

From the voltage curves of Fig. 6 the "motor unit territory" can be determined which would correspond to insertions at different distances from the centre of the motor unit. With a motor unit of a true territory extending over 9.3 mm, insertion of the electrode at a distance of three millimeters from the centre of the motor unit still gives a "territory" of 7.1 mm. Only when the electrode is inserted less than 1 mm from the periphery, the "territory" is measured substantially too small. Assuming random insertions the distribution of "territories" was calculated (Fig. 4 D). This distribution is clearly different from the experimentally found distributions shown in Fig. 4 A—C. Similar considerations as for the variation in territory apply to the variation in maximum voltage. Thus, in a given muscle the variation in territory and maximum voltage is mainly an expression of true differences between the motor units and is not due to eccentric insertions of the electrode. The differences between motor units in maximum voltage and territory are further discussed in terms of muscle fibre concentration and innervation ratio on p. 85.

Since the duration of the motor unit potential depends mainly on the width of the zone of innervation (BUCHTHAL, GULD and ROSENFALCK 1957 b), and since there was no correlation between mean duration and motor unit territory, the width of the zone of innervation is independent of motor unit territory.

Discussion.

In the study presented here the territory covered by the muscle fibres of a motor unit was determined from the distribution of action potentials containing spikes over a cross section of the muscle. The fibres of a motor unit were confined to a circular area in the extremity muscles investigated with the exception of the small muscles of the hand and the foot. In these, where leading off was only possible with the electrode inserted in the mediolateral plane of the muscle, the spread of the spike potentials of

the motor units exceeded the antero-posterior thickness of the muscle, indicating that the fibres of the individual motor unit are distributed over an oval-shaped area. Similar to our previous findings in the brachial biceps the extent of the territory found in the larger limb muscles indicates that the territory of a motor unit on the one hand is only a minor fraction of the total cross section of the muscles, but on the other hand is so large as to allow the intermingling of several different motor units. Five hundred to two thousand fibres of a motor unit (FEINSTEIN *et al.* 1955), when closely packed, would with an average fibre diameter of 50μ occupy an area of 1—2 mm diameter. Hence, in the area with a diameter of 5—10 mm, within which the fibres of a given motor unit are situated, 25 different motor units are represented. In fact, even with moderate effort, potentials from up to six different motor units have been recorded on the central leads of the multielectrode.

Maximum voltage varied for different motor units within the same muscle and average values of maximum voltage of the motor unit potentials were different in different types of muscle. An interpretation of these differences encounters difficulties, since the maximum voltage depends on several factors: Maximum voltage increases with the number of muscle fibres which contribute to the single spike of the motor unit potential ("subunit"). It increases also with decreasing distance between recording lead and active fibres and hence with a closer packing of the subunits. Thus a higher fibre concentration either due to a larger number of subunits per unit area or due to a larger number of muscle fibres per subunit is associated with a higher maximum voltage. However, also factors not related to fibre number determine maximum voltage. The higher the electrical impedance of the tissue surrounding the active subunit, the higher the voltage. Finally, the larger the area of the single leads of the multielectrode, the lower the voltage recorded. Hence, using the same multielectrode and assuming identical impedance, differences in maximum voltage in different motor units reflect differences in the muscle fibre concentration within the motor units. Thus, the correlation between maximum voltage and territory found in different motor units (Fig. 5) indicates that the larger the motor unit territory, the higher is the fibre concentration in the motor unit. Similarly, the differences in average maximum voltage in different types of muscles (Table I) indicate that the fibre concentration is largest within

the motor units of the intrinsic muscles of the hand and the foot.

If the territory of the motor unit is taken into account together with maximum voltage, conclusions may be drawn as to the innervation ratio in different muscles. In two muscles with equal maximum voltage, *i. e.* equal fibre concentration, the number of muscle fibres per motor unit is proportional to the area over which the fibres of the motor unit are distributed. Comparing for example the femoral biceps with the extensor digitorum communis, the ratio between average area per motor unit is $\left(\frac{8.8}{5.5}\right)^2 = 2.6$. Hence, the average fibre number per motor unit in the femoral biceps is 2.6 times that in the extensor digitorum communis.

In two muscles with equal territory, in for example the brachial biceps and the extensor digitorum communis, the fibre number per motor unit is greatest in the muscle with the highest maximum voltage. However, no quantitative estimate is possible as yet, since the relationship between maximum voltage and fibre concentration is unknown.

The territory and maximum voltage in normal muscle may serve as a basis for the demonstration of changes under pathological conditions. For the individual muscle six different motor units can usually be recorded without difficulty. If the mean value of the territory of these six motor units deviates more than two mm from the normal average (Table I), in muscles with a territory of 5—7.5 mm and more than 3—4 mm in muscles with 9—11 mm territory, it indicates a significant change with 95 per cent probability. Maximum voltage requires a forty per cent difference to be statistically significant with a 95 per cent probability.

Summary.

1. The spread of action potentials from motor units of different human muscles was studied by means of a twelve lead multielectrode. The occurrence of a positive-negative deflection in the potential of less than 0.2 msec duration indicated the presence of active muscle fibres in the immediate vicinity of the recording lead.

2. In this way the fibres of a motor unit were found to be confined to a circular area of 5—7 mm diameter for the muscles of the

upper extremities and of 7—10 mm in the lower extremities (Fig. 3, Table I). Such areas allow space for the fibres of 25 motor units each comprising 500—2,000 fibres.

3. Within the same muscle or the same type of muscle the diameter of the motor unit territory varied by a factor of four (Fig. 4). The maximum voltage within the motor unit increased linearly with the territory (Fig. 5).

4. The different average values of maximum voltage and territory in different muscles are interpreted in terms of differences in fibre concentration and total number of fibres per motor unit.

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Influence of Specific Group Inhibitors and Enzymes on Intrinsic Factor Activity.

By

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Abstract.

GRÄSBECK, RALPH. Influence of specific group inhibitors and enzymes on intrinsic factor activity. *Acta physiol. scand.* 1959. *45*. 88—95. — In order to elucidate the relationship of vitamin B_{12}-binding capacity and physiological intrinsic factor activity, human gastric juice and a hog stomach preparation were subjected to treatment with chemicals and enzymes. Thereafter the B_{12}-binding capacity and physiological activity were assayed by the use of Co^{60}-labeled vitamin B_{12}. In all cases where the B_{12}-binding power was lost, the intrinsic factor activity was also lost. In almost all cases where the binding power was retained the physiological activity also remained, only one exception being found. The results are interpreted to indicate that intrinsic factor (a part of the molecule or a subfactor in a multifactor system) binds B_{12}, and that a phenolic group may be contained in the binding structure. In addition, another part of the intrinsic factor seems to be involved in the physiological action but not in the binding process.

Several reports indicate that the binding of the vitamin in the form of a large-molecular complex is of importance in the intestinal absorption of vitamin B_{12}, and that it is likely that the capacity to bind B_{12} is a property of intrinsic factor (TERNBERG and EAKIN 1949, GRÄSBECK 1956, BUNGE and SCHILLING 1957). In the present

study pepsin-inactivated human gastric juice and a purified hog intrinsic factor preparation were treated with group blocking chemicals, enzymes, and u. v. light, and subsequently their B_{12}-binding capacity and clinical intrinsic factor activity were assayed. The influence on the B_{12}-binding capacity has been reported elsewhere (GRÄSBECK 1958 a). In the present investigation the influence on the clinical intrinsic factor activity was studied and compared to the action on the B_{12}-binding capacity.

Material and Methods.

The collection and processing of pepsin-inactivated human gastric juice as well as the conditions used during the treatment of intrinsic factor preparations with group blocking agents have been described in detail elsewhere (GRÄSBECK 1956, 1958 a). In addition to gastric juice, a "B_{12}-free" purified intrinsic factor preparation BFS 209 (obtained by courtesy of N. V. Organon, Oss, Holland) was used. It bound 0.27 μg B_{12} per mg and gave a good response in SCHILLING's urinary radioactivity test in 17 mg dose and a fair response in 5 mg dose.

Intrinsic factor activity was assayed in the following manner: The patients, who had pernicious anemia in hematologic remission and who had not been treated with intrinsic factor, fasted overnight. In the morning they were given 1 or 2 μg of Co^{60}-labeled cyanocobalamin ("Merck", diluted to a specific activity of about 160 μc/mg) in a glass containing pH 7.4 phosphate buffer. In the basal tests only this was administered by mouth, whereas in the actual tests, the intrinsic factor preparation studied was mixed with the radiovitamin before administration. 90 min after the oral dose 1 mg of nonlabeled B_{12} was injected intramuscularly and the urine was collected for 24 hours, counted from the beginning of the test. The patients were permitted to eat after the injection.

In all experiments where intrinsic factor preparations were treated in various ways, two identical intrinsic factor samples were used, usually about 30 ml of gastric juice or 8—15 mg of BFS 209 each. One sample received the specific treatment (enzyme digestion, irradiation, treatment with chemicals), whereas the other one served as a control, which was treated in an analogous way, the same pH, temperature, salt concentration, etc. being used, only the specific treatment being omitted. In the enzyme experiments, usually boiled enzyme was added to the controls.

In the intrinsic factor assays the same patient was subjected first to a basal test and then treated intrinsic factor preparations were given in varying order of administration, usually at 10—14 day intervals. If no increase was found in these two latter tests, a fourth test was performed in which a reference sample was given, containing untreated intrinsic factor.

The group blocking treatment, the effect of which was tested clinically, included iodination at alkaline pH, deamination with nitrous acid, acetylation with acetic anhydride, and irradiation with "monochromatic" 280 mμ u. v. light. The action of other specific group inhibitors was not tested clinically, because of their possible toxicity. The effect of these substances has been studied on gastrectomized rats (GRÄSBECK 1958 b).

The enzyme treatments included digestion with mucinase, chymotrypsin, pepsin, trypsin, and tyrosinase. The mucinase used was a "receptor-destroying enzyme" preparation from *Vibrio cholerae*. Monochromatic u. v. light was produced by filtering the light from a mercury lamp through various quartz and liquid filters. A small amount of other wavelengths than 280 mμ may have been present, however. When rigid precautions were taken to prevent entrance of other wavelengths, the action on the B_{12}-binding capacity was not so clearcut as observed before (GRÄSBECK 1958 a). For details of the experimental conditions and the preparations used, see GRÄSBECK (1958 a).

Results.

The results of the intrinsic factor assays are given in Fig. 1 and 2. For the sake of comparison, the effect of the treatment on the B_{12}-binding capacity, assayed with dialysis or ultrafiltration technique (GRÄSBECK 1958 a) is also reported in the figures.

Iodine was found to have a marked and unquestionable effect on both the B_{12}-binding and intrinsic factor activities. The observation was made, using both gastric juice and the hog stomach preparation. The nitrous acid-treated samples included two batches, one of which had been treated for 82 hours, which resulted in a 10 % decrease in B_{12}-binding capacity. This preparation lacked intrinsic factor activity, however, as did its control which had been kept at the same pH for an equally long time. The patient subsequently responded to a nontreated preparation. The other batch was treated for one hour, after which time its pH was raised by dialysis against sodium bicarbonate, still at 0° C. This sample bound as much B_{12} as its control, and apparently also retained its intrinsic factor activity.

Acetylation with acetic anhydride, which caused a 70—88 % drop in the color given by the ninhydrin reaction, caused practically no loss of B_{12}-binding power, but a total loss of physiological activity in all three cases studied. One control showed no clearcut effect; however, this patient responded very weakly to a nontreated sample of intrinsic factor (excretion 3 %). The u. v. light-treated

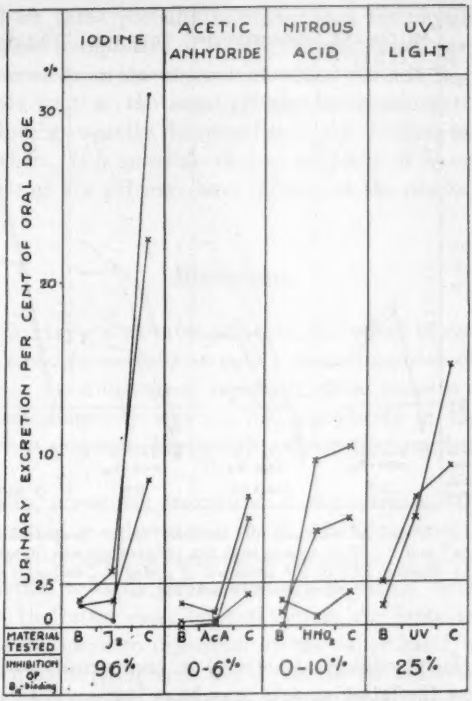

Fig. 1. Action of chemicals and u. v. light on intrinsic factor preparations. B = basal test (no intrinsic factor given), C = control preparation, ×———× = hog stomach preparation, •———• = human gastric juice.

samples used in the present experiments may have lost some intrinsic factor activity, and did lose 25 % of their B_{12}-binding capacity.

The enzyme experiments yielded the following results: Trypsin and the receptor-destroying enzyme had no effect on the B_{12}-binding power, and fairly certainly no effect on the intrinsic factor activity. This holds true regardless of whether digestion was performed in the presence or in the absence of added radiovitamin B_{12}. In three experiments tyrosinase lacked effect on the B_{12}-binding power in two cases and had a questionable effect in the third (10 % decrease). One of the two former samples showed no

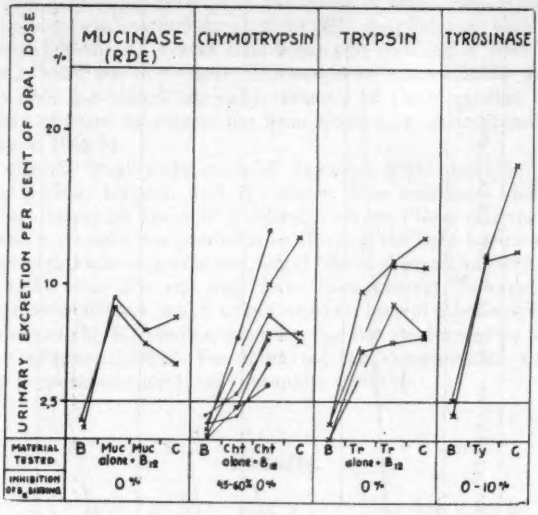

Fig. 2. Action of enzymes on intrinsic factor preparations. "alone" and "+ B_{12}" means that the preparation was digested in the absence and in the presence of added B_{12}, respectively. Other symbols as in Fig. 1.

change in the physiological activity in comparison with its control, whereas the latter sample may have lost some activity.

As reported before (GRÄSBECK 1958 a), the B_{12}-binding capacity decreased considerably when chymotrypsin digestion was performed, provided no B_{12} had been added before digestion. On the other hand, if radiovitamin B_{12} was added before digestion, no decrease in the amount of bound vitamin was observed. In five cases (hog stomach preparation in one case), which all were clinically active after digestion in presence of B_{12}, three had no clearcut intrinsic factor activity, one very low and one considerably decreased activity when digested in the absence of B_{12}. The hog stomach sample digested in presence of B_{12} was equally active as a nondigested sample. Two additional samples of the hog stomach preparation, digested in presence of B_{12}, were about as active as their nondigested controls.

In addition to these experiments, samples of human gastric juice and the hog stomach preparation were digested with crystalline pepsin. The results were somewhat confusing. Assays

performed on three patients indicated that the physiological activity was lost irrespective of whether the digestion was performed in the presence or in the absence of added vitamin B_{12}. However, the controls kept at the same pH also lost considerable activity. The B_{12}-binding capacity decreased strongly in some samples and little in others. It is possible that a tendency of bound vitamin to dissociate at low pH may have influenced the results.

Discussion.

Due to shortage of suitable patients, the effect of each kind of treatment could be assayed on only a limited number of patients. Therefore, in some instances, especially those cases in which the intrinsic factor activity was not lost completely or the controls elicited a low response, the results cannot be considered wholly conclusive.

In all cases, where the treatment of the intrinsic factor preparations resulted in a decrease in the B_{12}-binding power, the clinical activity also decreased. A remarkable parallelism is to be observed in the reaction towards iodination and digestion with chymotrypsin. In the latter case, both activities are protected by the addition of B_{12} prior to digestion; on the other hand, other substrates of chymotrypsin were found not to be protected by B_{12} (GRÄSBECK 1958 a). This finding is fairly strong evidence in favor of the concept that the same structure in the molecule, the one protected (covered up?) by B_{12}, is involved in the B_{12}-binding phenomenon and the physiological intrinsic factor activity. Further parallel behavior in the two activities was found in their reaction towards treatment with fluorodinitrobenzene (assayed on gastrectomized rats, GRÄSBECK 1958 b). As discussed before (GRÄSBECK 1958 a), it is tempting to interpret the results as indicating that the active structure contains a tyrosyl group (both chymotrypsin and pepsin hydrolyze bonds involving tyrosine, which also reacts with iodine and fluorodinitrobenzene). The absence of effect of tyrosinase does not necessarily contradict this interpretation, because tyrosinase has been found to lack effect on tyrosyl groups of several proteins (HAAS, SIZER and LOOFBOUROW 1951), apparently due to steric hindrance.

The absence of effect of trypsin is contrary to the findings of others (see WIJMENGA 1957). This is probably due to the use of

a more purified preparation in the present experiments. The finding speaks against the presence in the active structure of trypsin-sensitive bonds and amino acids involved in these (lysine, arginine). The same applies to the lack of effect of mucinase, which indicates lack of bonds between lactaminic acid and lactose and the like (see PIPER 1958). The possibility that steric inaccessibility may have protected the bonds must be kept in mind, however. Also in these instances the B_{12}-binding capacity and the intrinsic factor activity reacted in a parallel manner.

The absence of effect on the B_{12}-binding capacity of acetic anhydride and nitrous acid, and also trypsin, seems to indicate that an amino group in the protein is not involved in the binding of the vitamin. The two latter treatments also lacked effect on the physiological activity. However, acetic anhydride may have had effect. It is therefore possible that the molecule, or alternatively, one subfactor in an intrinsic factor system, contains an amino group, which is necessary for B_{12} absorption-promoting action but not for the binding of B_{12}. However, other explanations are also possible. The treatment was performed at high salt concentrations near the isoelectric point (ROLAND, MILLMAN and GIFFEE 1953) of the B_{12}-binding protein. In the nitrous acid experiments long-time treatment at low pH destroyed the clinical activity of the control, and as reported above, standing at low pH decreased the clinical activity of some additional preparations. "Simple denaturation" may therefore be responsible for the loss of activity. These questions require more thorough study. Still, the effect of acetic anhydride shows that under certain conditions the B_{12}-binding activity is retained when the clinical activity is lost. This is a phenomenon not infrequently encountered by those who have tried to purify the intrinsic factor (for references see GRÄSBECK 1956, WIJMENGA 1957). Considering the weight of evidence presented in favor of the concept that B_{12}-binding is involved in the absorption of B_{12} and the action of intrinsic factor, it would be hazardous to draw the conclusion that intrinsic factor does not bind B_{12}. However, it shows that the molecule or system must contain parts, which are necessary for the clinical action but not for the binding activity. Similar conclusions were drawn on the basis of previous studies (GRÄSBECK 1956). There is at present no evidence to indicate whether the two parts of the structure lodge in the same or different molecules.

Summary.

Human gastric juice and a hog stomach preparation were treated with various chemicals, u. v. light and enzymes. Thereafter the B_{12}-binding capacity and physiological intrinsic factor activity were assayed. In all cases where the B_{12}-binding power was lost, the intrinsic factor activity was also lost. In almost all cases where the binding power was retained the physiological activity also remained, only one exception being found. The results are interpreted to indicate that intrinsic factor (a part of the molecule or a subfactor in a multifactor system) binds B_{12}, and that a phenolic group may be contained in the binding structure. In addition, another part of the intrinsic factor seems to be involved in the physiological action but not in the binding process.

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